(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number : **0 614 977 A2**

(12) EUROPEAN PATENT APPLICATION

(21) Application number : **94301587.5**

(22) Date of filing : **07.03.94**

(51) Int. Cl.[5] : **C12N 15/12**, C07K 13/00, C12N 1/21, C12N 5/10, C07K 15/28, C12N 5/16, C12Q 1/68, A61K 37/02, A61K 48/00, C12P 21/08

(30) Priority : **05.03.93 US 27498**
01.07.93 US 85000

(43) Date of publication of application :
14.09.94 Bulletin 94/37

(84) Designated Contracting States :
AT BE CH DE DK ES FR GB GR IE IT LI LU MC NL PT SE

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(54) Huntingtin DNA, protein and uses thereof.

(57) A novel gene, *huntingtin*, is described, encoding huntingtin protein, recombinant vectors and hosts capable of expressing huntingtin. Methods for the diagnosis and treatment of Huntington's disease are also provided.

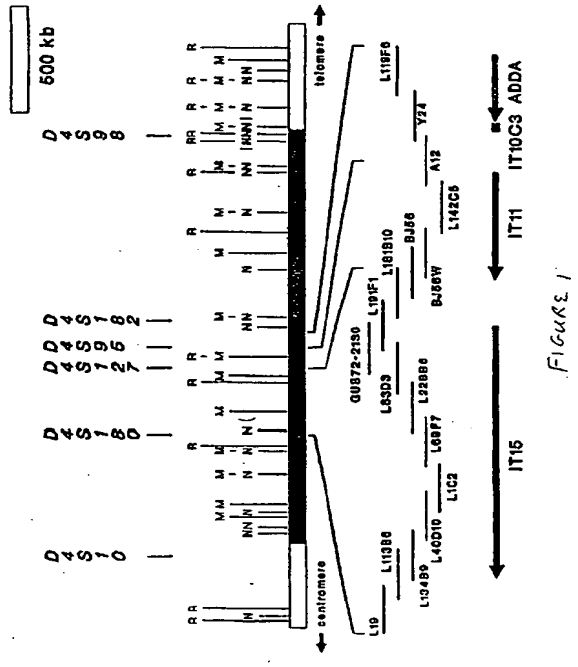

FIGURE 1

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Field of the Invention

The invention is in the field of the detection and treatment of genetic diseases. Specifically, the invention is directed to the *huntingtin* gene (also called the IT15 gene), huntingtin protein encoded by such gene, and the use of this gene and protein in assays (1) for the detection of a predisposition to develop Huntington's disease, (2) for the diagnosis of Huntington's disease (3) for the treatment of Huntington's disease, and (4) for monitoring the course of treatment of such treatment.

Background of the Invention

Huntington's disease (HD) is a progressive neurodegenerative disorder characterized by motor disturbance, cognitive loss and psychiatric manifestations (Martin and Gusella, *N. Engl. J. Med. 315*:1267-1276 (1986). It is inherited in an autosomal dominant fashion, and affects about 1/10,000 individuals in most populations of European origin (Harper, P.S. *et al.*, in *Huntington's disease*, W.B. Saunders, Philadelphia, 1991). The hallmark of HD is a distinctive choreic movement disorder that typically has a subtle, insidious onset in the fourth to fifth decade of life and gradually worsens over a course of 10 to 20 years until death. Occasionally, HD is expressed in juveniles typically manifesting with more severe symptoms including rigidity and a more rapid course. Juvenile onset of HD is associated with a preponderance of paternal transmission of the disease allele. The neuropathology of HD also displays a distinctive pattern, with selective loss of neurons that is most severe in the caudate and putamen regions of the brain. The biochemical basis for neuronal death in HD has not yet been explained, and there is consequently no treatment effective in delaying or preventing the onset and progression of this devastating disorder.

The genetic defect causing HD was assigned to chromosome 4 in 1983 in one of the first successes of linkage analysis using polymorphic DNA markers in man (Gusella *et al., Nature 306*:234-238 (1983). Since that time, we have pursued a location cloning approach to isolating and characterizing the HD gene based on progressively refining its localization (Gusella, *FASEB J. 3*:2036-2041 (1989); Gusella, *Adv. Hum. Genet. 20*:125-151 (1991)). Among other work, this has involved the generation of new genetic markers in the region by a number of techniques (Pohl *et al., Nucleic Acids Res. 16*:9185-9198 (1988); Whaley *et al., Somat. Cell. Mol. Genet. 17*:83-91 (1991); MacDonald *et al., J. Clin. Inv. 84*:1013-1016 (1989)), the establishment of genetic (MacDonald *et al., Neuron 3*:183-190(1989); Allitto *et al., Genomics 9*:104-112 (1991)) and physical maps of the implicated regions (Bucan *et al., Genomics 6*:1-15 (1990); Bates *et al., Nature Genet. 1*:180-187 (1992); Doucette-Stamm *et al., Somat. Cell Mol. Genet. 17*:471-480 (1991); Altherr *et al., Genomics 13*:1040-1046 (1992)), the cloning of the 4p telomere of an HD chromosome in a YAC clone (Bates *et al., Am. J. Hum. Genet. 46*:762-775 (1990); Youngman *et al., Genomics 14*:350-356 (1992)), the establishment of YAC [yeast artificial chromosome] (Bates *et al., Nature Genet. 1*:180-187 (1992)) and cosmid (Baxendale *et al.*, in preparation) contigs (a series of overlapping clones which together form a whole sequence) of the candidate region, as well as the analysis and characterization of a number of candidate genes from the region (Thompson *et al., Genomics 11*:1133-1142 (1991); Taylor *et al., Nature Genet. 2*:223-227 (1992); Ambrose *et al., Hum. Mol. Genet. 1*:697-703 (1992)). Analysis of recombination events in HD kindreds has identified a candidate region of 2.2 Mb, between D4S10 and D4S98 in 4p16.3, as the most likely position of the HD gene (MacDonald *et al., Neuron 3*:183-190 (1989); Bates *et al., Am. J. Hum. Genet. 49*:7-16 (1991); Snell *et al., Am. J. Hum. Genet. 51*:357-362 (1992)). Investigations of linkage disequilibrium between HD and DNA markers in 4p16.3 (Snell *et al., J. Med. Genet. 26*:673-675 (1989); Theilman *et al., J. Med. Genet. 26*:676-681 (1989)) have suggested that multiple mutations have occurred to cause the disorder (MacDonald *et al., Am. J. Hum. Genet. 49*:723-734 (1991)). However, haplotype analysis using multi-allele markers has indicated that at least 1/3 of HD chromosomes are ancestrally related (MacDonald *et al., Nature Genet. 1*: 99-103 (1992)). The haplotype shared by these HD chromosomes points to a 500 kb segment between D4S180 and D4S182 as the most likely site of the genetic defect.

Targeting this 500 kb region for saturation with gene transcripts, exon amplification has been used as a rapid method for obtaining candidate coding sequences (Buckler *et al., Proc. Natl. Acad. Sci. USA 88*:4005-4009 (1991)). This strategy has previously identified three genes: the a-adducin gene (ADDA) (Taylor *et al., Nature Genet. 2*:223-227 (1992)); a putative novel transporter gene (IT10C3) in the distal portion of this segment; and a novel G protein-coupled receptor kinase gene (IT11) in the central portion (Ambrose *et al., Hum. Mol. Genet. 1*:697-703 (1992)). However, no defects implicating any of these genes as the HD locus have been found.

Summary of the Invention

A large gene, termed herein "huntingtin" or "IT15," has been identified that spans about 210 kb and encodes a previously undescribed protein of about 348 kDa. The huntingtin reading frame contains a polymorphic $(CAG)_n$ trinucleotide repeat with at least 17 alleles in the normal population, varying from 11 to about 34 CAG copies. On HD chromosomes, the length of the trinucleotide repeat is substantially increased, for example, about 37 to at least 73 copies, and shows an apparent correlation with age of onset, the longest segments are detected in juvenile HD cases. The instability in length of the repeat is reminiscent of similar trinucleotide repeats in the fragile X syndrome and in myotonic dystrophy (Suthers *et al., J. Med. Genet. 29*:761-765 (1992)). The presence of an unstable, expandable trinucleotide repeat on HD chromosomes in the region of strongest linkage disequilibrium with the disorder suggests that this alteration underlies the dominant phenotype of HD, and that huntingtin encodes the HD gene.

The invention is directed to the protein huntingtin, DNA and RNA encoding this protein, and uses thereof.

Accordingly, in a first embodiment, the invention is directed to purified preparations of the protein huntingtin, preferably substantially cell-free.

In a further embodiment, the invention is directed to a recombinant construct containing DNA or RNA encoding huntingtin.

In a further embodiment, the invention is directed to a vector containing such huntingtin-encoding nucleic acid.

In a further embodiment, the invention is directed to a host transformed with such vector.

In a further embodiment, the invention is directed to a method for producing huntingtin from such recombinant host.

In a further embodiment, the invention is direct to a method for diagnosing Huntington's disease using such huntingtin DNA, RNA and/or protein.

In a further embodiment, the invention is directed to a method for treating Huntington's disease using such huntingtin DNA, RNA and/or protein.

In a further embodiment, the invention is directed to a method of gene therapy of a symptomatic or presymptomatic patient, such method comprising providing a functional *huntingtin* gene with a $(CAG)_n$ repeat of the normal range of 11-34 copies to the desired cells of such patient in need of such treatment, in a manner that permits the expression of the huntingtin protein provided by such gene, for a time and in a quantity sufficient to provide the huntingtin function to the cells of such patient.

In a further embodiment, the invention is directed to a method of gene therapy of a symptomatic or presymptomatic patient, such method comprising providing a functional *huntingtin* antisense gene to the desired cells of such patient in need of such treatment, in a manner that permits the expression of huntingtin antisense RNA provided by such gene, for a time and in a quantity sufficient to inhibit huntingtin mRNA expression in the cells of such patient.

In a further embodiment, the invention is directed to a method of gene therapy of a symptomatic or presymptomatic patient, such method comprising providing a functional *huntingtin* gene to the cells of such patient in need of such gene; in one embodiment the functional huntingtin gene contains a $(CAG)_n$ repeat size between 11-34 copies.

In a further embodiment, the invention is directed to a method for diagnosing Huntington's disease or a predisposition to develop Huntington's disease in a patient, such method comprising determining the number of $(CAG)_n$ repeats present in the huntingtin gene in such patient and especially in the affected tissue of such patient.

In a further embodiment, the invention is directed to a method for treating Huntington's disease in a patient, such method comprising decreasing the number of huntingtin $(CAG)_n$ repeats in the huntingtin gene in the desired cells of such patient.

Brief Description of the Drawings

FIGURE 1. Long-range restriction map of the *HD* candidate region. A partial long range restriction map of 4p16.3 is shown (adapted from Lin *et al., Somat. Cell Mol. Genet. 17*:481-488 (1991)). The HD candidate region determined by recombination events is depicted as a hatched line between *D4S10* and *D4S98*. The portion of the *HD* candidate region implicated as the site of the defect by linkage disequilibrium haplotype analysis (MacDonald *et al., Nature Genet. 1*:99-103 (1992) is shown as a filled box. Below the map schematic, the region from *D4S180* to *D4S182* is expanded to show the cosmid contig (averaging 40 kb/cosmid). The genomic coverage and where known the transcriptional orientation (arrow 5' to 3') of the huntingtin (IT15), IT11, IT10C3 and *ADDA* genes is also shown. Locus names above the map denote selected polymorphic markers that have

been used in HD families. The positions of *D4S127* and *D4S95* which form the core of haplotype in the region of maximum disequilibrium are also shown in the cosmid contig. Restriction sites are given for Not I (N), Mlu I (M) and Nru I (R). Sites displaying complete digestion are shown in boldface while sites subject to frequent incomplete digestion are shown as lighter symbols. Brackets around the "N" symbols indicate the presence of additional clustered Not I sites.

FIGURE 2. Northern blot analysis of the huntingtin (IT15) transcript. Results of the hybridization of IT15A to a Northern blot of RNA from normal (lane 1) and HD homozygous (lane 2 and 3) lymphoblasts are shown. A single RNA of about 11 kb was detected in all three samples, with slight apparent variations being due to unequal RNA concentrations. The HD homozygotes are independent, deriving from the large an American family (lane 2) and the large Venezuelan family (lane 3), respectively. The Venezuelan HD chromosome has a 4p16.3 haplotype of "5 2 2" defined by a $(GT)_n$ polymorphism at *D4S127* and VNTR and TaqI RFLPs at *D4S95*. The American homozygote carries the most common 4p16.3 haplotype found on HD chromosomes: "2 11 1" (MacDonald *et al., Nature Genet. 1*:99-103 (1992)).

FIGURE 3. Schematic of cDNA clones defining the IT15 transcript. Five cDNAs are represented under a schematic of the composite IT15 sequence. The thin line corresponds to untranslated regions. The thick line corresponds to coding sequence, assuming initiation of translation at the first Met codon in the open reading frame. Stars mark the positions of the following exon clones 5' to 3': DL83D3-8, DL83D3-1, DL228B6-3, DL228B6-5, DL228B6-13, DL69F7-3, DL178H4-6, DL118F5-U and DL134B9-U4.

The composite sequence was derived as follows. From 22 bases 3' to the putative initiator Met ATG, the sequence was compiled from the cDNA clones and exons shown. There are 9 bases of sequence intervening between the 3' end of IT16B and the 5' end of IT15B. These were by PCR amplification of first strand cDNA and sequencing of the PCR product. At the 5' end of the composite sequence, the cDNA clone IT16C terminates 27 bases upstream of the $(CAG)_n$. However, when IT16C was identified, we had already generated genomic sequence surrounding the $(CAG)_n$ in an attempt to generate new polymorphisms. This sequence matched the IT16C sequence, and extended it 337 bases upstream, including the apparent Met initiation codon.

FIGURE 4. Composite sequence of huntingtin (IT15)(SEQ ID NO:5 and SEQ ID NO:6). The composite DNA sequence of huntingtin (IT15) is shown (SEQ ID NO:5). The predicted protein product (SEQ ID NO:6) is shown below the DNA sequence, based on the assumption that translation begins at the first in-frame methionine of the long open reading frame.

FIGURE 5. DNA sequence analysis of the $(CAG)_n$ repeat. DNA sequence shown in panels 1, 2 and 3, demonstrates the variation in the $(CAG)_n$ repeat detected in normal cosmid L191F1 (1), cDNA IT16C (2), and *HD* cosmid GUS72-2130. Panels 1 and 3 were generated by direct sequencing of cosmid subclones using the following primer (SEQ ID NO:1):

5' GGC GGG AGA CCG CCA TGG CG 3'.

Panel 2 was generated using the pBSKII T7 primer (SEQ ID NO:2):

5' AAT ACG ACT CAC TAT AG 3'.

FIGURE 6. PCR analysis of the $(CAG)_n$ repeat in a Venezuelan HD sibship with some offspring displaying juvenile onset. Results of PCR analysis of a sibship in the Venezuela HD pedigree are shown. Affected individuals are represented by shaded symbols. Progeny are shown as triangles for confidentiality. AN1, AN2 and AN3 mark the positions of the allelic products from normal chromosomes. AE marks the range of PCR products from the *HD* chromosome. The intensity of background constant bands, which represent a useful reference for comparison of the above PCR products, varies with slight differences in PCR conditions. The PCR products from cosmids L191F1 and GUS72-2130 are loaded in lanes 12 and 13 and have 18 and 48 CAG repeats, respectively.

FIGURE 7. PCR analysis of the $(CAG)_n$ repeat in a Venezuelan HD sibship with offspring homozygous for the same *HD* haplotype. Results of PCR analysis of a sibship from the Venezuela HD pedigree in which both parents are affected by HD are shown. Progeny are shown as triangles for confidentiality and no HD diagnostic information is given to preserve the blind status of investigators in the Venezuelan Collaborative Group. AN1 and AN2 mark the positions of the allelic products from normal parental chromosomes. AE marks the range of PCR products from the *HD* chromosome. The PCR products from cosmids L191F1 and GUS72-2130 are loaded in lanes 29 and 30 and have 18 and 48 CAG repeats, respectively.

FIGURE 8. PCR analysis of the $(CAG)_n$ repeat in members of an American family with an individual homozygous for the major *HD* haplotype. Results of PCR analysis of members of an American family segregating the major HD haplotype. AN marks the range of normal alleles; AE marks the range of *HD* alleles. Lanes 1, 3,

4, 5, 7 and 8 represent PCR products from related *HD* heterozygotes. Lane 2 contains the PCR products from a member of the family homozygous for the same *HD* chromosome. Lane 6 contains PCR products from a normal individual. Pedigree relationships and affected status are not presented to preserve confidentiality. The PCR products from cosmids L191F1 and GUS72-2130 (which was derived from the individual represented in lane 2) are loaded in lanes 9 and 10 and have 18 and 48 CAG repeats, respectively.

FIGURES 9 and 10. PCR analysis of the $(CAG)_n$ repeat in two families with supposed new mutation causing HD. Results of PCR analysis of two families in which sporadic HD cases representing putative new mutants are shown. Individuals in each pedigree are numbered by generation (Roman numerals) and order in the pedigree. Triangles are used to protect confidentiality. Filled symbols indicate symptomatic individuals. The different chromosomes segregating in the pedigree have been distinguished by extensive typing with polymorphic markers in 4p16.3 and have been assigned arbitrary numbers shown above the gel lanes. The starred chromosomes (3 in Figure 9, 1 in Figure 10) represent the presumed *HD* chromosome. AN denotes the range of normal alleles; AE denotes the range of alleles present in affected individuals and in their unaffected relatives bearing the same chromosomes.

FIGURE 11. Comparison of $(CAG)_n$ Repeat Unit Number on Control and HD Chromosomes. Frequency distributions are shown for the number of $(CAG)_n$ repeat units observed on 425 HD chromosomes from 150 independent families, and from 545 control chromosomes.

FIGURE 12. Comparison of (CAG)n Repeat Unit Number on Maternally and Paternally Transmitted HD Chromosomes. Frequency distributions are shown for the 134 and 161 HD chromosomes from Figure 11 known to have been transmitted from the mother (Panel A) and father (Panel B), respectively. The two distributions differ significantly based on a t-test ($t_{272.3}$ =5.34, $p<0.0001$).

FIGURE 13. Comparison of $(CAG)_n$ Repeat Unit Number on HD Chromosomes from Three Large Families with Different HD Founders. Frequency distributions are shown for 75, 25 and 35 HD chromosomes from the Venezuelan HD family (Panel A) (Gusella, J.F., *et al., Nature 306*:234- 238 (1983); Wexler, N.S., *et al., Nature 326*:194-197 (1987)), Family Z (Panel B) and Family D (Panel C) (Folstein, S.E., *et al., Science 229*:776-779 (1985)), respectively. The Venezuelan distribution did not differ from the overall HD chromosome distribution in Figure 11 ($t_{79.7}$= 1.58, $p<0.12$). Both Family Z and Family D did produce distributions significantly different from the overall HD distribution ($t_{42.2}$=6.73, $p<0.0001$ and t_{458}=2.90, $p<0.004$, respectively).

Figure 14. Relationship of $(CAG)_n$ Repeat Length in Parents and Corresponding Progeny. Repeat length on the HD chromosome in mothers (Panel A) or fathers (Panel B) is plotted against the repeat length in the corresponding offspring. A total of 25 maternal transmissions and 37 paternal transmissions were available for typing.

FIGURE 15. Amplification of the HD $(CAG)_n$ Repeat From Sperm and Lymphoblast DNA. DNA from sperm (S) and lymphoblasts (L) for 5 members (pairs 1-5) of the Venezuelan HD pedigree aged 24-30 were used for PCR amplification of the HD $(CAG)_n$ repeat. The lower band in each lane derives from the normal chromosome.

FIGURE 16. Relationship of Repeat Unit Length with Age of Onset. Age of onset was established for 234 diagnosed HD gene carriers and plotted against the repeat length observed on both the HD and normal chromosomes in the corresponding lymphoblast lines.

Detailed Description of the Invention

In the following description, reference will be made to various methodologies known to those of skill in the art of molecular genetics and biology. Publications and other materials setting forth such known methodologies to which reference is made are incorporated herein by reference in their entireties as though set forth in full.

The IT15 gene described herein is a gene from the proximal portion of the 500 kb segment between human chromosome 4 markers *D4S180* and *D4S182*. The huntingtin gene spans about 210 kb of DNA and encodes a previously undescribed protein of about 348 kDa. The huntingtin reading frame contains a polymorphic $(CAG)_n$ trinucleotide repeat with at least 17 alleles in the normal human population, where the repeat number varies from 11 to about 34 CAG copies in such alleles. This is the gene of the human chromosome that, as shown herein, suffers the presence of an unstable, expanded number of CAG trinucleotide repeats in Huntington's disease patients, such that the number of CAG repeats in the huntingtin gene increases to a range of 37 to at least 86 copies. These results are the basis of a conclusion that the huntingtin gene encodes a protein called "huntingtin," and that in such huntingtin gene the increase in the number of CAG repeats to a range of greater than about 37 repeats is the alteration that underlies the dominant phenotype of Huntington's disease. As used herein huntingtin gene is also called the Huntington's disease gene.

It is to be understood that the description below is applicable to any gene in which a CAG repeat within the gene is amplified in an aberrant manner resulting in a change in the regulation, localization, stability or translatability of the mRNA containing such amplified CAG repeat that is transcribed from such gene.

I. Cloning Of Huntingtin DNA And Expression Of Huntingtin Protein

The identification of huntingtin DNA and protein as the altered gene in Huntington's disease patients is exemplified below. In addition to utilizing the exemplified methods and results for the identification of deletions of the *huntingtin* gene in Huntington's disease patients, and for the isolation of the native human *huntingtin* gene, the sequence information presented in Figure 4 represents a nucleic acid and protein sequence, that, when inserted into a linear or circular recombinant nucleic acid construct such as a vector, and used to transform a host cell, will provide copies of *huntingtin* DNA and huntingtin protein that are useful sources for the native *huntingtin* DNA and huntingtin protein for the methods of the invention. Such methods are known in the art and are briefly outlined below.

The process for genetically engineering the *huntingtin* coding sequence, for expression under a desired promoter, is facilitated through the cloning of genetic sequences which are capable of encoding such huntingtin protein. Such cloning technologies can utilize techniques known in the art for construction of a DNA sequence encoding the huntingtin protein, such as, for example, polymerase chain reaction technologies utilizing the *huntingtin* sequence disclosed herein to isolate the *huntingtin* gene anew, or an allele thereof that varies in the number of CAG repeats in such gene, or polynucleotide synthesis methods for constructing the nucleotide sequence using chemical methods. Expression of the cloned *huntingtin* DNA provides huntingtin protein.

As used herein, the term "genetic sequences" is intended to refer to a nucleic acid molecule of DNA or RNA, preferably DNA. Genetic sequences that are capable of being operably linked to DNA encoding huntingtin protein, so as to provide for its expression and maintenance in a host cell are obtained from a variety of sources, including commercial sources, genomic DNA, cDNA, synthetic DNA, and combinations thereof. Since the genetic code is universal, it is to be expected that any DNA encoding the huntingtin amino acid sequence of the invention will be useful to express huntingtin protein in any host, including prokaryotic (bacterial) hosts, eukaryotic hosts (plants, mammals (especially human), insects, yeast, and especially any cultured cell populations).

If it is desired to select anew a gene encoding huntingtin from a library that is thought to contain a *huntingtin* gene, such library can be screened and the desired gene sequence identified by any means which specifically selects for a sequence coding for the *huntingtin* gene or expressed huntingtin protein such as, for example, a) by hybridization (under stringent conditions for DNA:DNA hybridization) with an appropriate *huntingtin* DNA probe(s) containing a sequence specific for the DNA of this protein, such sequence being that provided in Figure 4 or a functional derivative thereof that is, a shortened form that is of sufficient length to identify a clone containing the *huntingtin* gene, or b) by hybridization-selected translational analysis in which native *huntingtin* mRNA which hybridizes to the clone in question is translated *in vitro* and the translation products are further characterized for the presence of a biological activity of huntingtin, or c) by immunoprecipitation of a translated huntingtin protein product from the host expressing the huntingtin protein.

When a human allele does not encode the identical sequence to that of Figure 4, it can be isolated and identified as being *huntingtin* DNA using the same techniques used herein, and especially PCR techniques to amplify the appropriate gene with primers based on the sequences disclosed herein. Many polymorphic probes useful in the fine localization of genes on chromosome 4 are known and available (see, for example, "ATCC/NIH Repository Catalogue of Human and Mouse DNA Probes and Libraries," fifth edition, 1991, pages 4-6. For example, a useful *D4S10* probe is clone designation pTV20 (ATCC 57605 and 57604); H5.52 (ATCC 61107 and 61106) and F5.53 (ATCC 61108).

Human chromosome 4-specific libraries are known in the art and available from the ATCC for the isolation of probes ("ATCC/NIH Repository Catalogue of Human and Mouse DNA Probes and Libraries," fifth edition, 1991, pages 72-73), for example, LL04NS01 and LL04NS02 (ATCC 57719 and ATCC57718) are useful for these purposes.

It is not necessary to utilize the exact vector constructs exemplified in the invention; equivalent vectors can be constructed using techniques known in the art. For example, the sequence of the huntingtin DNA is provided herein, (see Figure 4) and this sequence provides the specificity for the *huntingtin* gene; it is only necessary that a desired probe contain this sequence, or a portion thereof sufficient to provide a positive indication of the presence of the *huntingtin* gene.

Huntingtin genomic DNA may or may not include naturally occurring introns. Moreover, such genomic DNA can be obtained in association with the native *huntingtin* 5' promoter region of the gene sequences and/or with the native *huntingtin* 3' transcriptional termination region.

Such *huntingtin* genomic DNA can also be obtained in association with the genetic sequences which encode the 5' non-translated region of the *huntingtin* mRNA and/or with the genetic sequences which encode the *huntingtin* 3' non-translated region. To the extent that a host cell can recognize the transcriptional and/or translational regulatory signals associated with the expression of *huntingtin* mRNA and protein, then the

5' and/or 3' non-transcribed regions of the native *huntingtin* gene, and/or, the 5' and/or 3' non-translated regions of the huntingtin mRNA can be retained and employed for transcriptional and translational regulation.

Genomic DNA can be extracted and purified from any host cell, especially a human host cell possessing chromosome 4, by means well known in the art. Genomic DNA can be shortened by means known in the art, such as physical shearing or restriction digestion, to isolate the desired *huntingtin* gene from a chromosomal region that otherwise would contain more information than necessary for the utilization of the *huntingtin* gene in the hosts of the invention. For example, restriction digestion can be utilized to cleave the full-length sequence at a desired location. Alternatively, or in addition, nucleases that cleave from the 3'-end of a DNA molecule can be used to digest a certain sequence to a shortened form, the desired length then being identified and purified by polymerase chain reaction technologies, gel electrophoresis, and DNA sequencing. Such nucleases include, for example, Exonuclease III and *Bal31*. Other nucleases are well known in the art.

Alternatively, if it is known that a certain host cell population expresses huntingtin protein, then cDNA techniques known in the art can be utilized to synthesize a cDNA copy of the huntingtin mRNA present in such population.

For cloning the genomic or cDNA nucleic acid that encodes the amino acid sequence of the huntingtin protein into a vector, the DNA preparation can be ligated into an appropriate vector. The DNA sequence encoding huntingtin protein can be inserted into a DNA vector in accordance with conventional techniques, including blunt-ending or staggered-ending termini for ligation, restriction enzyme digestion to provide appropriate termini, filling in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and ligation with appropriate ligases. Techniques for such manipulations are well known in the art.

When the huntingtin DNA coding sequence and an operably linked promoter are introduced into a recipient eukaryotic cell (preferably a human host cell) as a non-replicating, non-integrating, molecule, the expression of the encoded huntingtin protein can occur through the transient (nonstable) expression of the introduced sequence.

Preferably the coding sequence is introduced on a DNA molecule, such as a closed circular or linear molecule that is capable of autonomous replication. If integration into the host chromosome is desired, it is preferable to use a linear molecule. If stable maintenance of the *huntingtin* gene is desired on an extrachromosomal element, then it is preferable to use a circular plasmid form, with the appropriate plasmid element for autonomous replication in the desired host.

The desired gene construct, providing a gene coding for the huntingtin protein, and the necessary regulatory elements operably linked thereto, can be introduced into a desired host cells by transformation, transfection, or any method capable of providing the construct to the host cell. A marker gene for the detection of a host cell that has accepted the *huntingtin* DNA can be on the same vector as the *huntingtin* DNA or on a separate construct for cotransformation with the huntingtin coding sequence construct into the host cell. The nature of the vector will depend on the host organism.

Suitable selection markers will depend upon the host cell. For example, the marker can provide biocide resistance, e.g., resistance to antibiotics, or heavy metals, such as copper, or the like.

Factors of importance in selecting a particular plasmid or viral vector include: the ease with which recipient cells that contain the vector can be recognized and selected from those recipient cells which do not contain the vector; the number of copies of the vector which are desired in a particular host; and whether it is desirable to be able to "shuttle" the vector between host cells of different species.

When it is desired to use *S. cerevisiae* as a host for a shuttle vector, preferred *S. cerevisiae* yeast plasmids include those containing the 2-micron circle, etc., or their derivatives. Such plasmids are well known in the art and are commercially available.

Oligonucleotide probes specific for the *huntingtin* sequence can be used to identify clones to huntingtin and can be designed *de novo* from the knowledge of the amino acid sequence of the protein as provided herein in Figure 4 or from the knowledge of the nucleic acid sequence of the DNA encoding such protein as provided herein in Figure 4 or of a related protein. Alternatively, antibodies can be raised against the huntingtin protein and used to identify the presence of unique protein determinants in transformants that express the desired cloned protein.

A nucleic acid molecule, such as DNA, is said to be "capable of expressing" a huntingtin protein if that nucleic acid contains expression control sequences which contain transcriptional regulatory information and such sequences are "operably linked" to the huntingtin nucleotide sequence which encode the huntingtin polypeptide.

An operable linkage is a linkage in which a sequence is connected to a regulatory sequence (or sequences) in such a way as to place expression of the sequence under the influence or control of the regulatory sequence. If the two DNA sequences are a coding sequence and a promoter region sequence linked to the 5' end of the coding sequence, they are operably linked if induction of promoter function results in the transcription of mRNA

encoding the desired protein and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the expression regulatory sequences to direct the expression of the protein, antisense RNA, or (3) interfere with the ability of the DNA template to be transcribed. Thus, a promoter region would be operably linked to a DNA sequence if the promoter was capable of effecting transcription of that DNA sequence.

The precise nature of the regulatory regions needed for gene expression can vary between species or cell types, but shall in general include, as necessary, 5' non-transcribing and 5' non-translating (non-coding) sequences involved with initiation of transcription and translation respectively, such as the TATA box, capping sequence, CAAT sequence, and the like, with those elements necessary for the promoter sequence being provided by the promoters of the invention. Such transcriptional control sequences can also include enhancer sequences or upstream activator sequences, as desired.

The vectors of the invention can further comprise other operably linked regulatory elements such as DNA elements which confer antibiotic resistance, or origins of replication for maintenance of the vector in one or more host cells.

In another embodiment, especially for maintenance of the vectors of the invention in prokaryotic cells, or in yeast *S. cerevisiae* cells, the introduced sequence is incorporated into a plasmid or viral vector capable of autonomous replication in the recipient host. Any of a wide variety of vectors can be employed for this purpose. In *Bacillus* hosts, integration of the desired DNA can be necessary.

Expression of a protein in eukaryotic hosts such as a human cell requires the use of regulatory regions functional in such hosts. A wide variety of transcriptional and translational regulatory sequences can be employed, depending upon the nature of the host. Preferably, these regulatory signals are associated in their native state with a particular gene which is capable of a high level of expression in the specific host cell, such as a specific human tissue type. In eukaryotes, where transcription is not linked to translation, such control regions may or may not provide an initiator methionine (AUG) codon, depending on whether the cloned sequence contains such a methionine. Such regions will, in general, include a promoter region sufficient to direct the initiation of RNA synthesis in the host cell.

If desired, the non-transcribed and/or non-translated regions 3' to the sequence coding for the huntingtin protein can be obtained by the above-described cloning methods. The 3'-non-transcribed region of the native human *huntingtin* gene can be retained for its transcriptional termination regulatory sequence elements, or for those elements which direct polyadenylation in eukaryotic cells. Where the native expression control sequences signals do not function satisfactorily in a host cell, then sequences functional in the host cell can be substituted.

It may be desired to construct a fusion product that contains a partial coding sequence (usually at the amino terminal end) of a first protein or small peptide and a second coding sequence (partial or complete) of the huntingtin protein at the carboxyl end. The coding sequence of the first protein can, for example, function as a signal sequence for secretion of the huntingtin protein from the host cell. Such first protein can also provide for tissue targeting or localization of the huntingtin protein if it is to be made in one cell type in a multicellular organism and delivered to another cell type in the same organism. Such fusion protein sequences can be designed with or without specific protease sites such that a desired peptide sequence is amenable to subsequent removal.

The expressed huntingtin protein can be isolated and purified from the medium of the host in accordance with conventional conditions, such as extraction, precipitation, chromatography, affinity chromatography, electrophoresis, or the like. For example, affinity purification with anti-huntingtin antibody can be used. A protein having the amino acid sequence shown in Figure 3 can be made, or a shortened peptide of this sequence can be made, and used to raised antibodies using methods well known in the art. These antibodies can be used to affinity purify or quantitate huntingtin protein from any desired source.

If it is necessary to extract huntingtin protein from the intracellular regions of the host cells, the host cells can be collected by centrifugation, or with suitable buffers, lysed, and the protein isolated by column chromatography, for example, on DEAE-cellulose, phosphocellulose, polyribocytidylic acid-agarose, hydroxyapatite or by electrophoresis or immunoprecipitation.

II. Use Of Huntingtin For Diagnostic And Treatment Purposes

It is to be understood that although the following discussion is specifically directed to human patients, the teachings are also applicable to any animal that expresses huntingtin and in which alteration of huntingtin, especially the amplification of CAG repeat copy number, leads to a defect in huntingtin gene (structure or function) or huntingtin protein (structure or function or expression), such that clinical manifectations such as those seen in Huntington's disease patients are found.

It is also to be understood that the methods referred to herein are applicable to any patient suspected of developing/having Huntington's disease, whether such condition is manifest at a young age or at a more advanced age in the patient's life. It is also to be understood that the term "patient" does not imply that symptoms are present, and patient includes any individual it is desired to examine or treat using the methods of the invention.

The diagnostic and screening methods of the invention are especially useful for a patient suspected of being at risk for developing Huntington's disease based on family history, or a patient in which it is desired to diagnose or eliminate the presence of the Huntington's disease condition as a causative agent behind a patient's symptoms.

It is to be understood that to the extent that a patient's symptoms arise due to the alteration of the CAG repeat copy numbers in the *huntingtin* gene, even without a diagnosis of Huntington's disease, the methods of the invention can identify the same as the underlying basis for such condition.

According to the invention, presymptomatic screening of an individual in need of such screening for their likelihood of developing Huntington's disease is now possible using DNA encoding the huntingtin gene of the invention, and specifically, DNA having the sequence of the normal human huntingtin gene. The screening method of the invention allows a presymptomatic diagnosis, including prenatal diagnosis, of the presence of an aberrant *huntingtin* gene in such individuals, and thus an opinion concerning the likelihood that such individual would develop or has developed Huntington's disease or symptoms thereof. This is especially valuable for the identification of carriers of altered huntingtin gene alleles where such alleles possess an increased number of CAG repeats in their huntingtin gene, for example, from individuals with a family history of Huntington's disease. Especially useful for the determination of the number of CAG repeats in the patient's *huntingtin* gene is the use of PCR to amplify such region or DNA blotting techniques.

For example, in the method of screening, a tissue sample would be taken from such individual, and screened for (1) the presence of the 'normal' human *huntingtin* gene, especially for the presence of a "normal" range of 11-34 CAG copies in such gene. The human *huntingtin* gene can be characterized based upon, for example, detection of restriction digestion patterns in 'normal' versus the patient's DNA, including RFLP analysis, using DNA probes prepared against the *huntingtin* sequence (or a functional fragment thereof) taught in the invention. Similarly, huntingtin mRNA can be characterized and compared to normal huntingtin mRNA (a) levels and/or (b) size as found in a human population not at risk of developing Huntington's disease using similar probes. Lastly, huntingtin protein can be (a) detected and/or (b) quantitated using a biological assay for huntingtin, for example, using an immunological assay and anti-huntingtin antibodies. When assaying huntingtin protein, the immunological assay is preferred for its speed. Methods of making antibody against the huntingtin are well known in the art.

An (1) aberrant *huntingtin* DNA size pattern, such as an aberrant *huntingtin* RFLP, and/or (2) aberrant huntingtin mRNA sizes or levels and/or (3) aberrant huntingtin protein levels would indicate that the patient has developed or is at risk for developing a huntingtin-associated symptom such as a symptom associated with Huntington's disease.

The screening and diagnostic methods of the invention do not require that the entire huntingtin DNA coding sequence be used for the probe. Rather, it is only necessary to use a fragment or length of nucleic acid that is sufficient to detect the presence of the huntingtin gene in a DNA preparation from a normal or affected individual, the absence of such gene, or an altered physical property of such gene (such as a change in electrophoretic migration pattern).

Prenatal diagnosis can be performed when desired, using any known method to obtain fetal cells, including amniocentesis, chorionic villous sampling (CVS), and fetoscopy. Prenatal chromosome analysis can be used to determine if the portion of chromosome 4 possessing the normal *huntingtin* gene is present in a heterozygous state, and PCR amplification or DNA blotting utilized for estimating the size of the CAG repeat in the *huntingtin* gene.

The huntingtin DNA can be synthesized, especially, the CAG repeat region can be amplified and, if desired, labeled with a radioactive or nonradioactive reporter group, using techniques known in the art (for example, *see* Eckstein, F., Ed., *Oligonucleotides and Analogues: A Practical Approach,* IRS Press at Oxford University Press, New York, 1992); and Kricka, L.J., Ed., *Nonisotopic DNA Probe Techniques,* Academic Press, San Diego, (1992)).

In one method of treating Huntington's disease in a patient in need of such treatment, functional *huntingtin* DNA is provided to the cells of such patient, preferably prior to such symptomatic state that indicates the death of many of the patient's neuronal cells which it is desired to target with the method of the invention. The replacement *huntingtin* DNA is provided in a manner and amount that permits the expression of the huntingtin protein provided by such gene, for a time and in a quantity sufficient to treat such patient. Many vector systems are known in the art to provide such delivery to human patients in need of a gene or protein missing from the

cell. For example, adenovirus or retrovirus systems can be used, especially modified retrovirus systems and especially herpes simplex virus systems. Such methods are provided for, in, for example, the teachings of Breakefield, X.A. *et al., The New Biologist 3*:203-218 (1991); Huang, Q. *et al., Experimental Neurology 115*:303-316 (1992), WO93/03743 and WO90/09441 each incorporated herein fully by reference. Methods of antisense strategies are known in the art (see, for example, *Antisense Strategies,* Baserga, R. *et al.,* Eds., Annals of the New York Academy of Sciences, volume 660, 1992).

In another method of treating Huntington's disease in a patient in need of such treatment, a gene encoding an expressible sequence that transcribes *huntingtin* antisense RNA is provided to the cells of such patient, preferably prior to such symptomatic state that indicates the death of many of the patient's neuronal cells which it is desired to target with the method of the invention. The replacement *huntingtin* antisense RNA gene is provided in a manner and amount that permits the expression of the antisense RNA provided by such gene, for a time and in a quantity sufficient to treat such patient, and especially in an amount to inhibit translation of the aberrant huntingtin mRNA that is being expressed in the cells of such patient. As above, many vector systems are known in the art to provide such delivery to human patients in need of a gene or protein which is altered in the patients' cells. For example, adenovirus or retrovirus systems can be used, especially modified retrovirus systems and especially herpes simplex virus systems. Such methods are provided for, in, for example, the teachings of Breakefield, X.A. *et al., The New Biologist 3*:203-218 (1991); Huang, Q. *et al., Experimental Neurology 115*:303-316 (1992), WO93/03743 and WO90/09441 each incorporated herein fully by reference.

Delivery of a DNA sequence encoding a functional huntingtin protein, such as the amino acid encoding sequence of Figure 4, will effectively replace the altered *huntingtin* gene of the invention, and inhibit, and/or stop and/or regress the symptoms that are the result of the interference to *huntingtin* gene expression due to an increased number of CAG repeats, such as 37 to 86 repeats in the *huntingtin* gene as compared to the 11-34 CAG repeats found in human populations not at risk for developing Huntington's disease.

Because Huntington's disease is characterized by a loss of neurons that is most severe in the caudate and putamen regions of the brain, the method of treatment of the invention is most effective when the replacement *huntingtin* gene is provided to the patient early in the course of the disease, prior to the loss of many neurons due to cell death. For that reason, presymptomatic screening methods according to the invention are important in identifying those individuals in need of treatment by the method of the invention, and such treatment preferably is provided while such individual is presymptomatic.

In a further method of treating Huntington's disease in a patient in need of such treatment such method provides an antagonist to the aberrant huntingtin protein in the cells of such patient.

Although the method is specifically described for DNA-DNA probes, it is to be understood that RNA possessing the same sequence information as the DNA of the invention can be used when desired.

For diagnostic assays, huntingtin antibodies are useful for quantitating and evaluating levels of huntingtin protein, and are especially useful in immunoassays and diagnostic kits.

In another embodiment, the present invention relates to an antibody having binding affinity to an huntingtin polypeptide, or a binding fragment thereof. In a preferred embodiment, the polypeptide has the amino acid sequence set forth in SEQ ID NO:6, or mutant or species variation thereof, or at least 7 contiguous amino acids thereof (preferably, at least 10, 15, 20, or 30 contiguous amino acids thereof). Those which bind selectively to huntingtin would be chosen for use in methods which could include, but should not be limited to, the analysis of altered huntingtin expression in tissue containing huntingtin.

The antibodies of the present invention include monoclonal and polyclonal antibodies, as well fragments of these antibodies. Antibody fragments which contain the idiotype of the molecule can be generated by known techniques. For example, such fragments include but are not limited to: the $F(ab')_2$ fragment; the Fab' fragments, and the Fab fragments.

Of special interest to the present invention are antibodies to huntingtin (or their functional derivatives) which are produced in humans, or are "humanized" (i.e. non-immunogenic in a human) by recombinant or other technology. Humanized antibodies may be produced, for example by replacing an immunogenic portion of an antibody with a corresponding, but non-immunogenic portion (i.e. chimeric antibodies) (Robinson, R.R. *et al.,* International Patent Publication PCT/US86/02269; Akira, K. *et al.,* European Patent Application 184,187; Taniguchi, M., European Patent Application 171,496; Morrison, S.L. *et al.,* European Patent Application 173,494; Neuberger, M.S. et al., PCT Application WO 86/01533; Cabilly, S. *et al.,* European Patent Application 125,023; Better, M. *et al., Science 240*:1041-1043 (1988); Liu, A.Y. *et al., Proc. Natl. Acad. Sci. USA 84*:3439-3443 (1987); Liu, A.Y. *et al., J. Immunol. 139*:3521-3526 (1987); Sun, L.K. *et al., Proc. Natl. Acad. Sci. USA 84*:214-218 (1987); Nishimura, Y. *et al., Canc. Res. 47:999-1005* (1987); Wood, C.R. *et al., Nature 314*:446-449 (1985)); Shaw *et al., J. Natl. Cancer Inst. 80*:1553-1559 (1988). General reviews of "humanized" chimeric antibodies are provided by Morrison, S.L. *(Science, 229*:1202-1207 (1985)) and by Oi, V.T. *et al., BioTechniques 4*:214 (1986)). Suitable "humanized" antibodies can be alternatively produced by CDR or CEA substitution

(Jones, P.T. *et al., Nature 321*:552-525 (1986); Verhoeyan *et al., Science 239*:1534 (1988); Beidler, C.B. *et al., J. Immunol. 141*:4053-4060 (1988)).

In another embodiment, the present invention relates to a hybridoma which produces the above-described monoclonal antibody, or binding fragment thereof. A hybridoma is an immortalized cell line which is capable of secreting a specific monoclonal antibody.

In general, techniques for preparing monoclonal antibodies and hybridomas are well known in the art (Campbell, *"Monoclonal Antibody Technology: Laboratory Techniques in Biochemistry and Molecular Biology,"* Elsevier Science Publishers, Amsterdam, The Netherlands (1984); St. Groth *et al., J. Immunol. Methods 35*:1-21 (1980)).

Any animal (mouse, rabbit, and the like) which is known to produce antibodies can be immunized with the selected polypeptide. Methods for immunization are well known in the art. Such methods include subcutaneous or interperitoneal injection of the polypeptide. One skilled in the art will recognize that the amount of polypeptide used for immunization will vary based on the animal which is immunized, the antigenicity of the polypeptide and the site of injection.

The polypeptide may be modified or administered in an adjuvant in order to increase the peptide antigenicity. Methods of increasing the antigenicity of a polypeptide are well known in the art. Such procedures include coupling the antigen with a heterologous protein (such as globulin or β-galactosidase) or through the inclusion of an adjuvant during immunization.

For monoclonal antibodies, spleen cells from the immunized animals are removed, fused with myeloma cells, and allowed to become monoclonal antibody producing hybridoma cells.

Any one of a number of methods well known in the art can be used to identify the hybridoma cell which produces an antibody with the desired characteristics. These include screening the hybridomas with an ELISA assay, western blot analysis, or radioimmunoassay (Lutz *et al., Exp.Cell Res. 175*:109-124 (1988)).

Hybridomas secreting the desired antibodies are cloned and the class and subclass is determined using procedures known in the art (Campbell, *Monoclonal Antibody Technology: Laboratory Techniques in Biochemistry and Molecular Biology, supra* (1984)).

For polyclonal antibodies, antibody containing antisera is isolated from the immunized animal and is screened for the presence of antibodies with the desired specificity using one of the above-described procedures.

In another embodiment of the present invention, the above-described antibodies are detectably labeled. Antibodies can be detectably labeled through the use of radioisotopes, affinity labels (such as biotin, avidin, and the like), enzymatic labels (such as horse radish peroxidase, alkaline phosphatase, and the like) fluorescent labels (such as FITC or rhodamine, and the like), paramagnetic atoms, and the like. Procedures for accomplishing such labeling are well-known in the art, for example, see (Sternberger *et al., J. Histochem. Cytochem. 18*:315 (1970); Bayer *et al., Meth. Enzym. 62*:308 (1979); Engval *et al., Immunol. 109*:129 (1972); Goding, *J. Immunol. Meth. 13*:215 (1976)). The labeled antibodies of the present invention can be used for *in vitro, in vivo*, and *in situ* assays to identify cells or tissues which express a specific peptide.

In another embodiment of the present invention the above-described antibodies are immobilized on a solid support. Examples of such solid supports include plastics such as polycarbonate, complex carbohydrates such as agarose and sepharose, acrylic resins and such as polyacrylamide and latex beads. Techniques for coupling antibodies to such solid supports are well known in the art (Weir *et al., "Handbook of Experimental Immunology"* 4th Ed., Blackwell Scientific Publications, Oxford, England, Chapter 10 (1986); Jacoby *et al., Meth. Enzym. 34* Academic Press, N.Y. (1974)). The immobilized antibodies of the present invention can be used for *in vitro, in vivo*, and *in situ* assays as well as in immunochromotography.

Furthermore, one skilled in the art can readily adapt currently available procedures, as well as the techniques, methods and kits disclosed above with regard to antibodies, to generate peptides capable of binding to a specific peptide sequence in order to generate rationally designed antipeptide peptides, for example see Hurby *et al.*, "Application of Synthetic Peptides: Antisense Peptides", In *Synthetic Peptides, A User's Guide*, W.H. Freeman, NY, pp. 289-307 (1992), and Kaspczak *et al., Biochemistry 28*:9230-8 (1989).

Anti-peptide peptides can be generated in one of two fashions. First, the anti-peptide peptides can be generated by replacing the basic amino acid residues found in the huntingtin peptide sequence with acidic residues, while maintaining hydrophobic and uncharged polar groups. For example, lysine, arginine, and/or histidine residues are replaced with aspartic acid or glutamic acid and glutamic acid residues are replaced by lysine, arginine or histidine.

The manner and method of carrying out the present invention can be more fully understood by those of skill by reference to the following examples, which examples are not intended in any manner to limit the scope of the present invention or of the claims directed thereto.

Examples

The gene causing Huntington's disease has been mapped in 4p16.3 but has previously eluded identification. The invention uses haplotype analysis of linkage disequilibrium to spotlight a small segment of 4p16.3 as the likely location of the defect. A new gene, *huntingtin (IT15),* isolated using cloned "trapped" exons from a cosmid contig of the target area contains a polymorphic trinucleotide repeat that is expanded and unstable on HD chromosomes. A $(CAG)_n$ repeat longer than the normal range of about 11 to about 34 copies was observed on HD chromosomes from all 75 disease families examined, comprising a wide range of ethnic backgrounds and 4p16.3 haplotypes. The $(CAG)_n$ repeat, which varies from 37 to at least 86 copies on HD chromosomes appears to be located within the coding sequence of a predicted about 348 kDa protein that is widely expressed but unrelated to any known gene. Thus, the Huntington's disease mutation involves an unstable DNA segment, similar to those described in fragile X syndrome and myotonic dystrophy, acting in the context of a novel 4p16.3 gene to produce a dominant phenotype.

The following protocols and experimental details are referenced in the examples that follow.

HD Cell Lines. Lymphoblast cell lines from HD families of varied ethnic backgrounds used for genetic linkage and disequilibrium studies (Conneally *et al., Genomics 5*:304-308 (1989); MacDonald *et al., Nature Genet. 1*:99-103 (1992)) have been established (Anderson and Gusella, *In Vitro 20*:856-858 (1984)) in the Molecular Neurogenetics Unit, Massachusetts General Hospital, over the past 13 years. The Venezuelan HD pedigree is an extended kindred of over 10,000 members in which all affected individuals have inherited the *HD* gene from a common founder (Gusella *et al., Nature 306*:234-238 (1983); Gusella *et al., Science 225*:1320-1326 (1984); Wexler *et al., Nature 326*:194-197 (1987)).

DNA/RNA Blotting. DNA was prepared from cultured cells and DNA blots prepared and hybridized as described (Gusella *et al., Proc. Natl. Acad. Sci. USA 76*:5239-5243 (1979); Gusella *et al., Nature 306*:234-238 (1983)). RNA was prepared and Northern blotting performed as described in Taylor *et al., Nature Genet. 3*:223-227 (1992).

Construction of Cosmid Contig. The initial construction of the cosmid contig was by chromosome walking from cosmids L19 and BJ56 (Allitto *et al.,* Genomics 9:104-112 (1991); Lin *et al., Somat. Cell Mol. Genet. 17*:481-488 (1991)). Two libraries were employed, a collection of Alu-positive cosmids from the reduced cell hybrid H39-8C10 (Whaley *et al., Som. Cell Mol. Genet. 17*:83-91 (1991)) and an arrayed flow-sorted chromosome 4 cosmid library (NM87545) provided by the Los Alamos National Laboratory. Walking was accomplished by hybridization of whole cosmid DNA, using suppression of repetitive and vector sequences, to robot-generated high density filter grids (Nizetic, D. *et al., Proc. Natl. Acad. Sci. USA 88*:3233-3237 (1991); Lehrach, H. *et al.,* in *Genome Analysis: Genetic and Physical Mapping, Volume 1,* Davies, K.E. *et al.,* Ed., Cold Spring Harbor Laboratory Press, 1991, pp. 39-81). Cosmids L1C2, L69F7, L228B6 and L83D3 were first identified by hybridization of YAC clone YGA2 to the same arrayed library (Bates *et al., Nature Genet. 1*:180-187 (1992); Baxendale *et al., Nucleic Acids Res. 19*:6651 (1991)). *HD* cosmid GUS72-2130 was isolated by standard screening of a GUS72 cosmid library using a single-copy probe. Cosmid overlaps were confirmed by a combination of clone-to-clone and clone-to-genomic hybridizations, single-copy probe hybridizations and restriction mapping.

cDNA Isolation and Characterization. Exon probes were isolated and cloned as described (Buckler *et al., Proc. Natl. Acad. Sci. USA 88*:4005-4009 (1991)). Exon probes and cDNAs were used to screen human lambdaZAPII cDNA libraries constructed from adult frontal cortex, fetal brain, adenovirus transformed retinal cell line RCA, and liver RNA. cDNA clones, PCR products and trapped exons were sequenced as described (Sanger *et al., Proc. Natl. Acad. Sci. USA 74*:5463-5467 (1977)). Direct cosmid sequencing was performed as described (McClatchey *et al., Hum. Mol. Genet. 1*:521-527 (1992)). Database searches were performed using the BLAST network service of National Center for Biotechnology Information (Altschul *et al., J. Mol. Biol. 215*:403-410 (1990)).

PCR Assay of the $(CAG)_n$ Repeat. Genomic primers (SEQ ID NO:3 and SEQ ID NO:4) flanking the $(CAG)_n$ repeat are:

5′ ATG AAG GCC TTC GAG TCC CTC AAG TCC TTC 3′

and

5′ AAA CTC ACG GTC GGT GCA GCG GCT CCT CAG 3′.

PCR amplification was performed in a reaction volume of 25 µl using 50 ng of genomic DNA, 5 µg of each primer, 10 mM Tris, pH 8.3, SmM KCl, 2mM $MgCl_2$, 200 µM dNTPs, 10% DMSO, 0.1 unit Perfectmatch (Stra-

tagene), 2.5 μCi ^{32}P-dCTP (Amersham) and 1.25 units Taq polymerase (Boehringer Mannheim). After heating to 94°C for 1.5 minutes, the reaction mix was cycled according to the following program: 40 X [1'@94°C;1'@60°C;2'@72°C]. 5 μl of each PCR reaction was diluted with an equal volume of 95 % formamide loading dye and heat denatured for 2 min. at 95°C. The products were resolved on 5 % denaturing polyacrylamide gels. The PCR product from this reaction using cosmid L191F1 (CAG_{18}) as template was 247 bp. Allele sizes were estimated relative to a DNA sequencing ladder, the PCR products from sequenced cosmids, and the invariant background bands often present on the gel. Estimates of allelic variation were obtained by typing unrelated individuals of largely Western European ancestry, and normal parents of affected HD individuals from various pedigrees.

Typing of HD and normal chromosomes in Examples 5-8. HD chromosomes were derived from symptomatic individuals and "at risk" individuals known to be gene carriers by linkage marker analysis. All HD chromosomes were from members of well-characterized HD families of varied ethnic backgrounds used previously for genetic linkage and disequilibrium studies (MacDonald, M.E., *et al., Nature Genet. 1*:99-103 (1992); Conneally, P.M., *et al., Genomics 5*:304-308 (1989)). Three of the 150 families used were large pedigrees, each descended from a single founder. The large Venezuelan HD pedigree is an extended kindred of over 13,000 members from which we typed 75 HD chromosomes (Gusella, J.F., *et al., Nature 306*:234-238 (1983); Wexler, N.S., *et al., Nature 326*:194-197 (1987)). Two other large families that have been described previously as Family Z and Family D, provided 25 and 35 HD chromosomes, respectively (Folstein, S.E., *et al., Science 229*:776-779 (1985)). Normal chromosomes were taken from married-ins in the HD families and from unrelated normal individuals from non-HD families. The DNA tested for all individuals except four was prepared from lymphoblastoid cell lines or fresh blood (Gusella, J.F., *et al., Nature 306*:234-238 (1983); Anderson and Gusella, *In Vitro 20*:856-858 (1984)). In the exceptional cases, DNA was prepared from frozen cerebellum. No difference in the characteristics of the PCR products were observed between lymphoblastoid, fresh blood, or brain DNAs. For five members of the Venezuelan pedigree aged 24-30, we also prepared DNA by extracting pelleted sperm from semen samples. The length of the HD gene $(CAG)_n$ repeat for all DNAs was assessed using polymerase chain reaction amplification.

Statistical analysis as set forth in Examples 5-8. Associations between repeat lengths and onset age were assessed by Pearson correlation coefficient and by multivariate regression to assess higher order associations. Comparisons of the distributions of repeat length for all HD chromosomes and those for individual families were made by analysis of variance and t-test contrasts between groups. The 95 % confidence bands were computed around the regression line utilizing the general linear models procedure of SAS (SAS Institute Inc., SAS/STAT User's Guide, Version 6, Fourth Edition, Volume 2 (SAS Institute Inc., Cary, N.C., pp. 846, 1989)).

Example 1

Application of Exon Amplification to Obtain Trapped Cloned Exons

The *HD* candidate region defined by discrete recombination events in well-characterized families spans 2.2 Mb between *D4S10* and *D4S98* as shown in Figure 1. The 500 kb segment between *D4S180* and *D4S182* displays the strongest linkage disequilibrium with *HD*, with about 1/3 of disease chromosomes sharing a common haplotype, anchored by multi-allele polymorphisms at *D4S127* and *D4S95* (MacDonald *et al., Nature Genet. 1*:99-103 (1992)). Sixty-four overlapping cosmids spanning about 480 kb from *D4S180* to a location between *D4S95* and *D4S182* have been isolated by a combination of information from YAC (Baxendale *et al., Nucleic Acids Res. 19*:6651 (1991)) and cosmid probe hybridization to high density filter grids of a chromosome 4 specific library, as well as additional libraries covering this region. Sixteen of these cosmids providing the complete contig are shown in Figure 1. We have previously used exon amplification to identify *ADDA*, the α-adducin locus, IT10C3, a novel putative transporter gene, and IT11, a novel G protein-coupled receptor kinase gene in the region distal to *D4S127* (Figure 1).

We have now applied the exon amplification technique to cosmids from the region of the contig proximal to *D4S127*. This procedure produces "trapped" exon clones, which can represent single exons, or multiple exons spliced together and is an efficient method of obtaining probes for screening cDNA libraries. Individual cosmids were processed, yielding 9 exon clones in the region from cosmids L134B9 to L181B10.

Two non-overlapping cDNAs were initially isolated using exon probes. IT15A was obtained by screening a transformed adult retinal cell cDNA library with exon clone DL118F5-U. IT16A was isolated by screening an adult frontal cortex cDNA library with a pool of three exon clones, DL83D3-8, DL83D3-1, and DL228B6-3. By Northern blot analysis, we discovered that IT15A and IT16A are in fact different portions of the same large approximately 10-11 kb transcript. Figure 2 shows an example of a Northern blot containing RNA from lymphoblastoid cell lines representing a normal individual and 2 independent homozygotes for *HD* chromosomes

of different haplotypes. The same approximately 10-11 kb transcript was also detected in RNA from a variety of human tissues (liver, spleen, kidney, muscle and various regions of adult brain).

IT15A and IT16A were used to "walk" in a number of human tissue cDNA libraries in order to obtain the full-length transcript. Figure 3 shows a representation of 5 cDNA clones which define the IT15 transcript, under a schematic of the composite sequence derived as described in the legend. Figure 3 also displays the locations on the composite sequence of the 9 trapped exon clones.

The composite sequence of IT15, containing the entire predicted coding sequence, spans 10,366 bases including a tail of 18 A's as shown in Figure 4. An open reading frame of 9,432 bases begins with a potential initiator methionine codon at base 316, located in the context of an optimal translation initiation sequence. An in-frame stop codon is located 240 bases upstream from this site. The protein product of IT15 is predicted to be a 348 kDa protein containing 3,144 amino acids. Although the first Met codon in the long open reading frame has been chosen as the probably initiator codon, we cannot exclude that translation does not actually begin at a more 3' Met codon, producing a smaller protein.

Example 2

Polymorphic Variation of the $(CAG)_n$ Trinucleotide Repeat

Near its 5' end, the IT15 sequence contains 21 copies of the triplet CAG, encoding glutamine (Figure 5). When this sequence was compared with genomic sequences that are known to surround simple sequence repeats (SSRs) in 4p16.3, it was found that normal cosmid L191F1 had 18 copies of the triplet indicating that the $(CAG)_n$ repeat is polymorphic (Figure 5). Primers from the genomic sequence flanking the repeat were chosen to establish a PCR assay for this variation. In the normal population, this SSR polymorphism displays at least 17 discrete alleles (Table 1) ranging from about 11 to about 34 repeat units. Ninety-eight percent of the 173 normal chromosomes tested contained repeat lengths between 11 and 24 repeats. Two chromosomes were detected in the 25-30 repeat range and 2 normal chromosomes had 33 and 34 repeats respectively. The overall heterozygosity on normal chromosome was 80%. Based on sequence analysis of three clones, it appears that the variation is based entirely on the $(CAG)_n$, but the potential for variation of the smaller downstream $(CCG)_7$ which is also included in the PCR product, is also present.

Example 3

Instability of the Trinucleotide Repeat on HD chromosomes

Sequence analysis of cosmid GUS72-2130, derived from a chromosome with the major *HD* haplotype (see below), revealed 48 copies of the trinucleotide repeat, far greater than the largest normal allele (Figure 5). When the PCR assay was applied to *HD* chromosomes, a pattern strikingly different from the normal variation was observed. *HD* heterozygotes contained one discrete allelic product in the normal size range, and one PCR product of much larger size, suggesting that the $(CAG)_n$ repeat on *HD* chromosomes is expanded relative to normal chromosomes.

Figure 6 shows the patterns observed when the PCR assay was performed on lymphoblast DNA from a selected nuclear family in a large Venezuelan HD kindred. In this family, DNA marker analysis has shown previously that the *HD* chromosome was transmitted from the father (lane 2) to seven children (lanes 3, 5, 6, 7, 8, 10 and 11). The three normal chromosomes present in this mating yielded a PCR product in the normal size range (AN1, AN2, AN3) that was inherited in a Mendelian fashion. The *HD* chromosome in the father yielded a diffuse, "fuzzy"-appearing PCR product slightly smaller than the 48 repeat product of the non-Venezuelan HD cosmid. Except for the DNA in lane 5 which did not PCR amplify and in lane 11 which displayed only a single normal allele, each of the affected children's DNAs yielded a fuzzy PCR product of a different size (AE), indicating instability of the *HD* chromosome $(CAG)_n$ repeat. Lane 6 contained an *HD*- specific product slightly smaller than or equal to that of the father's DNA. Lanes 3, 7, 10 and 8, respectively, contained *HD*-specific PCR products of progressively larger size. The absence of an *HD*-specific PCR product in lane 11 suggested that this child's DNA possessed a $(CAG)_n$ repeat that was too long to amplify efficiently. This was verified by Southern blot analysis in which the expanded *HD* allele was easily detected and estimated to contain up to 100 copies of the repeat. Notably, this child had juvenile onset of HD at the very early age of 2 years. The onset of HD in the father was in his early 40s, typical of most adult HD patients in this population. The onset ages of children represented by lanes 3, 7, 10 and 8 were 26, 25, 14 and 11 years, respectively, suggesting a rough correlation between age at onset of HD and the length of the $(CAG)_n$ repeat on the *HD* chromosome. In keeping with this trend, the offspring represented in lane 6 with the fewest repeats remained asymptomatic

when last examined at age of 30.

Figure 7 shows PCR analysis for a second sibship from the Venezuelan pedigree in which both parents are *HD* heterozygotes carrying the same *HD* chromosome based on DNA marker studies. Several of the offspring are *HD* homozygotes (lanes 6+7, 10+11, 13+14, 17+18, 23+24) as reported previously (Wexler *et al., Nature 326*:194-197 (1987)). Each parent's DNA contained one allele in the normal range (AN1, AN2) which was transmitted in a Mendelian fashion. The *HD*-specific products (AE) from the DNA of both parents and children were all much larger than the normal allelic products and also showed extensive variation in mean size. A neurologic diagnosis for the offspring in this pedigree was not provided to maintain the blind status of investigators involved in the ongoing Venezuela HD project, although age of onset again appears to parallel repeat length. Paired samples under many of the individual symbols represent independent lymphoblast lines initiated at least one year apart. The variance between paired samples was not as great as between the different individuals, suggesting that the major differences in size of the PCR products resulted from meiotic transmission. Of special note is the result obtained in lanes 13 and 14. This *HD* homozygote's DNA yielded one PCR product larger and one smaller than the *HD*-specific PCR products of both parents.

To date, we have tested 75 independent HD families, representing all different reported in MacDonald *et al., Nature Genet. 1*:99-103 (1992)) and a wide range of ethnic backgrounds. In all 75 cases, a PCR product larger than the normal size range was produced from the *HD* chromosome. The sizes of the *HD*-specific products ranged from 42 repeat copies to more than 66 copies, with a few individuals failing to yield a product because of the extreme length of the repeat. In these cases, Southern blot analysis revealed an increase in the length of an EcoRI fragment with the largest allele approximating 100 copies of the repeat. Figure 8 shows the variation detected in members of an American family of Irish ancestry in which the major *HD* haplotype is segregating. Cosmid GUS72-2130 was cloned from the *HD* homozygous individual whose DNA was amplified in lane 2. As was observed in the Venezuelan HD pedigree (Figures 6 and 7), which segregates the disorder with a different 4p16.3 haplotype, the *HD*-specific PCR products for this family display considerable size variation.

Example 4

New Mutations to HD

The mutation rate in HD has been reported to be very low. To test whether the expansion of the $(CAG)_n$ repeat is the mechanism by which new *HD* mutations occur, two pedigrees with sporadic cases of HD have been examined in which intensive searching failed to reveal a family history of the disorder. In these cases, pedigree information sufficient to identify the same chromosomes in both the affected individual and unaffective relatives was gathered. Figures 9 and 10 show the results of PCR analysis of the $(CAG)_n$ repeat in these families. The chromosomes in each family were assigned an arbitrary number based on typing for a large number of RFLP and SSR markers in 4p16.3 defining distinct haplotypes and the presumed *HD* chromosome is starred.

In family #1, HD first appeared in individual II-3 who transmitted the disorder to III-1 along with chromosome 3*. This same chromosome was present in II-2, an elderly unaffected individual. PCR analysis revealed that chromosome 3* from II-2 produced a PCR product at the extreme high end of the normal range (about 36 CAG copies). However, the $(CAG)_n$ repeat on the same chromosome in II-3 and III-1 had undergone sequential expansions to about 44 and about 46 copies, respectively. A similar result was obtained in Family #2, where the presumed *HD* mutant III-2 had a considerably expanded repeat relative to the same chromosome in II-1 and III-1 (about 49 vs. about 33 CAG copies). In both family #1 and family #2, the ultimate *HD* chromosome displays the marker haplotype characteristic of 1/3 of all *HD* chromosomes, suggesting that this haplotype may be predisposed to undergoing repeat expansion.

Discussion

The discovery of an expanded, unstable trinucleotide repeat on *HD* chromosomes within the *IT15* gene is the basis for utilizing this gene as the *HD* gene of the invention. These results are consistent with the interpretation that HD constitutes the latest example of a mutational mechanism that may prove quite common in human genetic disease. Elongation of a trinucleotide repeat sequence has been implicated previously as the cause of three quite different human disorders, the fragile X syndrome, myotonic dystrophy and spino-bulbar muscular atrophy. The initial observations of repeat expansion in HD indicate that this phenomenon shares features in common with each of these disorders.

In the fragile X syndrome, expression of a constellation of symptoms that includes mental retardation and

a fragile site at Xq27.3 is associated with expansion of a $(CGG)_n$ repeat thought to be in the 5' untranslated region of the *FMR1* gene (Fu *et al., Cell 67*:1047-1058 (1991); Kremer *et al., Science 252*:1711-1714(1991); Verkerk *et al., Cell 65*:904-914 (1991)). In myotonic dystrophy, a dominant disorder involving muscle weakness with myotonia that typically present in early adulthood, the unstable trinucleotide repeat, $(CTG)_n$, is located in the 3' untranslated region of the mysotonin protein kinase gene (Aslanidis *et al., Nature 355*:548-551 (1992); Brook *et al., Cell 68*:799-808 (1992); Buxton *et al., Nature 355*:547-548 (1992); Fu *et al., Science 255*:1256-1259 (1992); Harley *et al., Lancet 339*:1125-1128 (1992); Mahadevan *et al., Science 255*:1253-1255 (1992)). The unstable $(CAG)_n$ repeat in HD may be within the coding sequence of the IT15 gene, a feature shared with spino-bulbar muscular atrophy, an X-linked recessive adult-onset disorder of the motor neurons caused by expansion of a $(CAG)_n$ repeat in the coding sequence of the androgen receptor gene (LaSpada *et al., Nature 352*:77-79 (1991)). The repeat length in both the fragile X syndrome and myotonic dystrophy tends to increase in successive generations, sometimes quite dramatically. Occasionally, decreases in the average repeat length are observed (Fu *et al., Science 255*:1256-1259 (1992); Yu *et al., Am. J. Hum. Genet. 50*:968-980 (1992); Bruner *et al., N. Engl. J. Med.*:476-480 (1993)). The *HD* trinucleotide repeat is also unstable, usually expanding when transmitted to the next generation, but contracting on occasion. In HD, as in the other disorders, change in copy number occurs in the absence of recombination. Compared with the fragile X syndrome, myotonic dystrophy, and HD, the instability of the disease allele in spino-bulbar muscular atrophy is more limited, and dramatic expansions of repeat length have not been seen (Biancalana *et al., Hum. Mol. Genet. 1*:255-258 (1992)).

Expansion of the repeat length in myotonic dystrophy is associated with a particular chromosomal haplotype, suggesting the existence of a primordial predisposing mutation (Harley *et al., Am. J. Hum. Genet. 49*:68-75 (1991); Harley *et al., Nature 355*:545-546 (1992); Ashizawa, *Lancet 338*:642-643 (1991); and Epstein (1991)). In the fragile X syndrome, there may be a limited number of ancestral mutations that predispose to increases in trinucleotide repeat number (Richards *et al., Nature Genet. 1*:257-260 (1992); Oudet *et al., Am. J. Hum. Genet. 52*:297-304 (1993)). The linkage disequilibrium analysis used to identify IT15 indicates that there are several haplotypes associated with HD, but that at least 1/3 of *HD* chromosomes are ancestrally related (MacDonald *et al., Nature Genet. 1*:99-103 (1992)). These data, combined with the reported low rate of new mutation to *HD* (Harper, *J. Med. Genet. 89*:365-376 (1992)), suggest that expansion of the trinucleotide repeat may only occur on select chromosomes. The analysis of two families presented herein, in which new mutation was supposed to have occurred, is consistent with the view that there may be particular normal chromosomes that have the capacity to undergo expansion of the repeat into the *HD* range. In each of these families, a chromosome with a $(CAG)_n$ repeat length in the upper end of the normal range was segregating on a chromosome whose 4p16.3 haplotype matched the most common haplotype seen on *HD* chromosomes and the clinical appearance of HD in these two cases was associated with expansion of the trinucleotide repeat.

The recent application of haplotype analysis to explore the linkage disequilibrium on *HD* chromosomes pointed to a portion of a 2.2 Mb candidate region defined by the majority of recombination events described in HD pedigrees (MacDonald *et al., Nature Genet 1*:99-103 (1992)). Previously, the search for the gene was confounded by three matings in which the genetic inheritance pattern was inconsistent with the remainder of the family (MacDonald *et al., Neuron 3*:183-190 (1989b); Prichard *et al., Am. J. Hum. Genet. 50*:1218-1230 (1992)). These matings produced apparently affected HD individuals despite the inheritance of only normal alleles for markers throughout 4p16.3, effectively excluding inheritance of the *HD* chromosome present in the rest of the pedigree. Using PCR assay disclosed above, each of these families was tested and it was determined that like other HD kindreds, an expanded allele segregates with *HD* in affected individuals of all three pedigrees. However, an expanded allele was not present in those specific individuals with the inconsistent 4p16.3 genotypes. Instead, these individuals displayed the normal alleles expected based on analysis of other markers in 4p16.3. It is conceivable that these inconsistent individuals do not, in fact, have HD, but some other disorder. Alternatively, they might represent genetic mosaics in which the *HD* allele is more heavily represented and/or more expanded in brain tissue than in the lymphoblast DNA used for genotyping.

The capacity to monitor directly the size of the trinucleotide repeat in individuals "at risk" for HD provides significant advantages over current methods, eliminating the need for complicated linkage analyses, facilitating genetic counseling, and extending the applicability of presymptomatic and prenatal diagnosis to "at risk" individuals with no living affected relatives. however, it is of the utmost importance that the current internationally accepted guidelines and counseling protocols for testing those "at risk" continue to be observed, and that samples from unaffected relatives should not be tested inadvertently or without full consent. In the series of patients examined in this study, there is an apparent correlation between repeat length and age of onset of the disease, reminiscent of that reported in myotonic dystrophy (Harley *et al., Lancet 339*:1125-1128 (1992); Tsilfidis *et al., Nature Genet. 1*:192-195 (1992)). The largest *HD* trinucleotide repeat segments were found in juvenile onset cases, where there is a known preponderance of male transmission (Merrit *et al., Excerpta Medica*, Amsterdam, pp. 645-650 (1969)).

The expression of fragile X syndrome is associated with direct inactivation of the *FMR1* gene (Pierretti *et al., Cell 66*:817-822 (1991); DeBoulle *et al., Nature Genet. 3*:31-35 (1993)). The recessive inheritance pattern of spino-bulbar muscular atrophy suggests that in this disorder, an inactive gene product is produced. In myotonic dystrophy, the manner in which repeat expansion leads to the dominant disease phenotype is unknown. There are numerous possibilities for the mechanism of pathogenesis of the expanded trinucleotide repeat in HD. Without intending to be held to this theory, nevertheless notice can be taken that since Wolf-Hirschhorn patients hemizygous for 4p16.3 do not display features of HD, and IT15 mRNA is present in *HD* homozygotes, the expanded trinucleotide repeat does not cause simple inactivation of the gene containing it. The observation that the phenotype of *HD* is completely dominant, since homozygotes for the disease allele do not differ clinically from heterozygotes, has suggested that HD results from a gain of function mutation, in which either the mRNA product or the protein product of the disease allele would have some new property, or be expressed inappropriately (Wexler *et al., Nature 326*:194-197 (1987); Myers *et al., Am. J. Hum. Genet. 45*:615-618 (1989)). If the expanded trinucleotide repeat were translated, the consequences on the protein product would be dramatic, increasing the length of the poly-glutamine stretch near the N-terminus. It is possible, however, that despite the presence of an upstream Met codon, the normal translational start occurs 3' to the $(CAG)_n$ repeat and there is no poly-glutamine stretch in the protein product. In this case, the repeat would be in the 5' untranslated region and might be expected to have its dominant effect at the mRNA level. The presence of an expanded repeat might directly alter regulation, localization, stability or translatability of the mRNA containing it, and could indirectly affect its counterpart from the normal allele in *HD* heterozygotes. Other conceivable scenarios are that the presence of an expanded repeat might alter the effective translation start site for the *HD* transcript, thereby truncating the protein, or alter the transcription start site for the IT15 gene, disrupting control of mRNA expression. Finally, although the repeat is located within the IT15 transcript, the possibility that it leads to HD by virtue of an action on the expression of an adjacent gene cannot be excluded.

Despite this final caveat, it is consistent with the above results and most likely that the trinucleotide repeat expansion causes HD by its effect, either at the mRNA or protein level, on the expression and/or structure of the protein product of the IT15 gene, which has been named huntingtin. Outside of the region of the triplet repeat, the IT15 DNA sequence detected no significant similarity to any previously reported gene in the GenBank database. Except for the stretches of glutamine and proline near the N-terminus, the amino acid sequence displayed no similarity to known proteins, providing no conspicuous clues to huntingtin's function. The poly-glutamine and poly-proline region near the N-terminus detect similarity with a large number of proteins which also contain long stretches of these amino acids. It is difficult to assess the significance of such similarities, although it is notable that many of these are DNA binding proteins and that huntingtin does have a single leucine zipper motiff at residue 1,443. Huntingtin appears to be widely expressed, and yet cell death in HD is confined to specific neurons in particular regions of the brain.

TABLE 1. COMPARISON OF HD AND NORMAL REPEAT SIZES

RANGE OF ALLELE SIZES (#REPEATS)	NORMAL CHROMOSOMES NUMBER AND FREQUENCY		HD CHROMOSOMES NUMBER AND FREQUENCY	
≥ 48	0	0	44	0.59
42-47	0	0	30	0.41
30-41	2	0.01	0	0
25-30	2	0.01	0	0
≤ 24	169	0.98	0	0
TOTAL	173	1.00	74	1.0

Example 5

Distribution of Trinucleotide Repeat Lengths on Normal and HD Chromosomes

The number of copies of the HD triplet repeat has been examined in a total of 425 HD chromosomes from 150 independent families and compared with the copy number of the HD triplet repeat of 545 normal chromosomes. The results are displayed in Figure 11. Two non-overlapping distributions of repeat length were observed, wherein the upper end of the normal range and the lower end of the HD range were separated by 3 repeat units. The normal chromosomes displayed 24 alleles producing PCR products ranging from 11 to 34 repeat units, with a median of 19 units (mean 19.71, s.d. 3.21). The HD chromosomes yielded 54 discrete PCR products corresponding to repeat lengths of 37 to 86 units, with a median of 45 units (mean 46.42, s.d. 6.68).

Of the HD chromosomes, 134 and 161 were known to be maternally or paternally-derived, respectively. To investigate whether the sex of the transmitting parent might influence the distribution of repeat lengths, these two sets of chromosomes were plotted separately in Figure 12. The maternally-derived chromosomes displayed repeat lengths ranging from 37 to 73 units, with a median of 44 (mean 44.93, s.d. 5.14). The paternally-derived chromosomes had 37 to 86 copies of the repeat unit, with a median of 48 units (mean 49.14, s.d. 8.27). However, a higher proportion of the paternally-derived HD chromosomes had repeat lengths greater than 55 units (16% vs. 2%), suggesting the possibility of a differential effect of paternal versus maternal transmission.

The data set used excluded chromosomes from a few clinically diagnosed individuals who have previously been shown not to have inherited the HD chromosome by DNA marker linkage studies (MacDonald, M.E., *et al., Neuron 3*:183-190 (1989); Pritchard, C., *et al., Am. J. Hum. Genet. 50*:1218-1230 (1992)). These individuals have repeat lengths well within the normal range. Their disease manifestations have not been explained, and they may represent phenocopies of HD. Regardless of the mechanism involved, the occurrence at low frequency of such individuals within known HD families must be considered if diagnostic conclusions are based solely on repeat length.

The control data set also excludes a number of chromosomes from phenotypically normal individuals who are related to "spontaneous" cases of HD or "new mutations". Chromosomes from these individuals who are not clinically affected and have no family history of the disorder cannot be designated as HD. However, these chromosomes cannot be classified as unambiguously normal because they are essentially the same chromo-

some as that of an affected relative, the diagnosed "spontaneous" HD proband, except with respect to repeat length. The lengths of repeat found on these ambiguous chromosomes (34-38 units) span the gap between the control and HD distributions, confounding a decision on the status of any individual with a repeat in the high normal to low HD range.

Example 6

Instability of the Trinucleotide Repeat

The data in Figure 11 combine repeat lengths from 150 different HD families representing many potentially independent origins of the defect. To examine the variation in repeat lengths on sets of HD chromosomes known to descend from a common founder, the data from three large HD kindreds (Gusella, J.F., *et al., Nature 306*:234-238 (1983); Wexler, N.S., *et al., Nature 326*:194-197 (1987); Folstein, S.E., *et al., Science 229*:776-779 (1985)) with different 4p16.3 haplotypes (MacDonald, M.E., *et al., Nature Genet. 1*:99-103 (1992)), typed for 75, 25 and 35 individuals, respectively, were separated. Despite the single origin of the founder HD chromosome within each pedigree, members of the separate pedigrees display a wide range of repeat lengths (Figure 13). This instability of the HD chromosome repeat is most prominent in members of a large Venezuelan HD kindred (panel A) In which the common HD ancestor has produced 10 generations of descendants, numbering over 13,000 individuals. The distribution of repeat lengths in this sampling of the Venezuelan pedigree (median 46, mean 48.26, s.d. 9.3) is not significantly different from that of the larger sample of HD chromosomes from all families. Panels B and C display results for two extended families in which HD was introduced more recently than in the Venezuelan kindred. These families have been reported to exhibit different age of onset distributions and varied phenotypic features of HD (Folstein, S.E., *et al., Science 229*:776-779 (1985)). Both revealed extensive repeat length variation, with a median of 41 and 49 repeat units, respectively. The distribution of repeat lengths in the members of the family in Panel B was significantly different from the distribution of all HD chromosome repeat lengths ($p<0.0001$), with a smaller mean of 42.04 repeat units (s.d. 2.82). The repeat distribution from HD chromosomes of Panel C was also significantly different from the total data set ($p<0.004$), but with a higher mean of 49.80(s.d. 5.86).

Example 7

Parental Source Effects on Repeat Length Variation

For 62 HD chromosomes in Figure 11, the length of the trinucleotide repeat also could be examined on the corresponding parental HD chromosome. In 20 of 25 maternal transmissions, and in 31 of 37 paternal transmissions, the repeat length was altered, indicating considerable instability. A similar phenomenon was not observed for normal chromosomes, where more than 500 meiotic transmissions revealed no changes in repeat length, although the very existence of such a large number of normal alleles suggests at least a low degree. of instability.

Figure 14 shows the relationship between the repeat lengths on the HD chromosomes in the affected parent and corresponding progeny. For the 20 maternally-inherited chromosomes on which the repeat length was altered, 13 changes were increases in length and 7 were decreases. Both increases and decreases involved changes of less than 5 repeat units and the overall correlation between the mother's repeat length and that of her child was $r=0.95$ ($p<0.0001$). The average change in repeat length in the 25 maternal transmissions was an increase of 0.4 repeats.

On paternally-derived chromosomes, the 31 transmissions in which the repeat length changes comprised 26 length increases and 5 length decreases. Although the decreases in size were only slightly smaller than those observed on maternally-derived chromosomes, ranging from 1 to 3 repeat units, the increases were often dramatically larger. Thus, the correlation of the repeat length in the father with that of his offspring was only $r=0.35$ ($p<0.04$). The average change in the 37 paternal transmissions was an increase of 9 repeat units. The maximum length increase observed through paternal transmission was 41 repeat units, a near doubling of the parental repeat.

For both male and female transmissions, there was no correlation between the size of the parental repeat and either the magnitude or frequency of changes.

To determine whether the variation in the length of the repeat observed through male transmission of HD chromosomes is reflected in the male germ cells, we amplified the repeat from sperm DNA and from DNA of the corresponding lymphoblast from 5 HD gene carriers. The results, shown in Figure 15, reveal striking differences between the lymphoblast and sperm DNA for the HD chromosome repeat, but not for the repeat on

the normal chromosome. All the sperm donors are members of the Venezuelan HD family and range in age from 24 to 30 years. Individuals 1 and 2 are siblings with HD chromosome repeat lengths based on lymphoblast DNA of 45 and 52, respectively. Individuals 3 and 4 are also siblings, with HD repeat lengths of 46 and 49, respectively. Individual 5, from a different sibship than either of the other two pairs, has an HD repeat of 52 copies. In all 5 cases, the PCR amplification of sperm DNA and lymphoblast DNA yielded identical products from the normal chromosome. However, in comparison with lymphoblast DNA, the HD gene from sperm DNA yielded a diffuse array of products. In 3 of the 5 cases (2,4 and 5), the diffuse array spread to much larger allelic products than the corresponding lymphoblast product. Subject 2 showed the greatest range of expansion, with the sperm DNA product extending to over 80 repeat units. Interestingly, the 3 individuals displaying the greatest variation have the longest repeats and are currently symptomatic. The other two donors have shorter repeat lengths in the HD range, and remain at risk at this time.

The striking difference in the high repeat length range (>55) between HD chromosomes transmitted from the father and those transmitted from the mother indicated a potential parental source effect. When this was examined directly, the HD chromosome repeat length changed in about 85% of transmissions. Most changes involved a fluctuation of only a few repeat units, with larger increases occurring only in male transmissions. The greater size increases in male transmission appear to be caused by particular instability of the HD trinucleotide repeat during male gametogenesis, based on the amplification of the repeat from sperm DNA.

Example 8

Relationship between Repeat Length and Age of Onset

Increased repeat length might correlate with a reduced age of onset of HD. Accordingly, age of onset data was determined for 234 of the individuals represented in Figure 11. Figure 16 displays the repeat lengths found on the HD and normal chromosomes of these individuals relative to their age of onset. Indeed, age of onset is inversely correlated with the HD repeat length. A Pearson correlation coefficient of $r=-.75$, $p<0.0001$ was obtained assuming a linear relationship between age of onset and repeat length. When a polynomial function was used, a better fit was obtained ($R^2=0.61$, $F=121.45$), suggesting a higher order association between age of onset and repeat length.

There is considerable variation in the age of onset associated with any specific number of repeat units, particularly for trinucleotide repeats in the 37-52 unit zone (88% of HD chromosomes) where onset ranged from 15 to 75 years. In this range, a linear relationship between age of onset and repeat length provided as good a fit as a higher order relationship. The 95 % confidence interval surrounding the predicted regression line was estimated at ±18 years. In the 37 to 52 unit range, the association of repeat length to onset age is only half as strong as in the overall distribution ($r=-0.40$, $p<.0001$), indicating that much of the predictive power is contributed by repeats longer than 52 units. In this increased range, onset is likely to be very young and consequently not relevant to most persons seeking testing.

For the 178 cases in the 37-52 repeat unit range for which it was possible to subdivide the data set based on parental origin of the HD gene, multivariate regression analysis suggested a significant effect of parental origin on age of onset ($p<0.05$) independent of repeat length in this range. HD gene carriers from maternal transmissions had an average age of onset two years later than those from paternal transmissions.

In both univariate and multivariate analyses, no association between age of onset and the repeat length on the normal chromosome was detected, either in the total data set, or when it was subdivided into chromosomes of maternal or paternal origin.

All publications mentioned hereinabove are hereby incorporated in their entirety by reference.

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be appreciated by one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention and appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: THE GENERAL HOSPITAL CORPORATION
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(ii) TITLE OF INVENTION: Huntingtin DNA, Protein And Uses Thereof

(iii) NUMBER OF SEQUENCES: 6

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) 7th March 1994

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 08/085,000
(B) FILING DATE: 01 JULY 1993

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 08/027,498
(B) FILING DATE: 05 MARCH 1993

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GGCGGGAGAC CGCCATGGCG 20

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AATACGACTC ACTATAG 17

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATGAAGGCCT TCGAGTCCCT CAAGTCCTTC 30

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AAACTCACGG TCGGTGCAGC GGCTCCTCAG 30

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10366 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 316..9748

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
TTGCTGTGTG AGGCAGAACC TGCGGGGGCA GGGGCGGGCT GGTTCCCTGG CCAGCCATTG      60

GCAGAGTCCG CAGGCTAGGG CTGTCAATCA TGCTGGCCGG CGTGGCCCCG CCTCCGCCGG     120

CGCGGCCCCG CCTCCGCCGG CGCACGTCTG GGACGCAAGG CGCCGTGGGG GCTGCCGGGA     180

CGGGTCCAAG ATGGACGGCC GCTCAGGTTC TGCTTTTACC TGCGGCCCAG AGCCCCATTC     240

ATTGCCCCGG TGCTGAGCGG CGCCGCGAGT CGGCCCGAGG CCTCCGGGGA CTGCCGTGCC     300

GGGCGGGAGA CCGCC ATG GCG ACC CTG GAA AAG CTG ATG AAG GCC TTC GAG       351
                 Met Ala Thr Leu Glu Lys Leu Met Lys Ala Phe Glu
                   1               5                  10

TCC CTC AAG TCC TTC CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG       399
Ser Leu Lys Ser Phe Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln
          15                  20                  25

CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAA CAG CCG CCA CCG CCG       447
Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln Pro Pro Pro Pro
      30                  35                  40

CCG CCG CCG CCG CCG CCT CCT CAG CTT CCT CAG CCG CCG CCG CAG GCA       495
Pro Pro Pro Pro Pro Pro Pro Gln Leu Pro Gln Pro Pro Pro Gln Ala
 45                  50                  55                  60

CAG CCG CTG CTG CCT CAG CCG CAG CCG CCC CCG CCG CCG CCC CCG CCG       543
Gln Pro Leu Leu Pro Gln Pro Gln Pro Pro Pro Pro Pro Pro Pro Pro
                   65                  70                  75

CCA CCC GGC CCG GCT GTG GCT GAG GAG CCG CTG CAC CGA CCA AAG AAA       591
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Pro Pro Gly Pro Ala Val Ala Glu Glu Pro Leu His Arg Pro Lys Lys
                80                  85                  90

GAA CTT TCA GCT ACC AAG AAA GAC CGT GTG AAT CAT TGT CTG ACA ATA      639
Glu Leu Ser Ala Thr Lys Lys Asp Arg Val Asn His Cys Leu Thr Ile
        95                  100                 105

TGT GAA AAC ATA GTG GCA CAG TCT GTC AGA AAT TCT CCA GAA TTT CAG      687
Cys Glu Asn Ile Val Ala Gln Ser Val Arg Asn Ser Pro Glu Phe Gln
    110                 115                 120

AAA CTT CTG GGC ATC GCT ATG GAA CTT TTT CTG CTG TGC AGT GAT GAC      735
Lys Leu Leu Gly Ile Ala Met Glu Leu Phe Leu Leu Cys Ser Asp Asp
125                 130                 135                 140

GCA GAG TCA GAT GTC AGG ATG GTG GCT GAC GAA TGC CTC AAC AAA GTT      783
Ala Glu Ser Asp Val Arg Met Val Ala Asp Glu Cys Leu Asn Lys Val
                145                 150                 155

ATC AAA GCT TTG ATG GAT TCT AAT CTT CCA AGG TTA CAG CTC GAG CTC      831
Ile Lys Ala Leu Met Asp Ser Asn Leu Pro Arg Leu Gln Leu Glu Leu
            160                 165                 170

TAT AAG GAA ATT AAA AAG AAT GGT GCC CCT CGG AGT TTG CGT GCT GCC      879
Tyr Lys Glu Ile Lys Lys Asn Gly Ala Pro Arg Ser Leu Arg Ala Ala
        175                 180                 185

CTG TGG AGG TTT GCT GAG CTG GCT CAC CTG GTT CGG CCT CAG AAA TGC      927
Leu Trp Arg Phe Ala Glu Leu Ala His Leu Val Arg Pro Gln Lys Cys
    190                 195                 200

AGG CCT TAC CTG GTG AAC CTT CTG CCG TGC CTG ACT CGA ACA AGC AAG      975
Arg Pro Tyr Leu Val Asn Leu Leu Pro Cys Leu Thr Arg Thr Ser Lys
205                 210                 215                 220

AGA CCC GAA GAA TCA GTC CAG GAG ACC TTG GCT GCA GCT GTT CCC AAA     1023
Arg Pro Glu Glu Ser Val Gln Glu Thr Leu Ala Ala Ala Val Pro Lys
                225                 230                 235

ATT ATG GCT TCT TTT GGC AAT TTT GCA AAT GAC AAT GAA ATT AAG GTT     1071
Ile Met Ala Ser Phe Gly Asn Phe Ala Asn Asp Asn Glu Ile Lys Val
            240                 245                 250

TTG TTA AAG GCC TTC ATA GCG AAC CTG AAG TCA AGC TCC CCC ACC ATT     1119
Leu Leu Lys Ala Phe Ile Ala Asn Leu Lys Ser Ser Ser Pro Thr Ile
        255                 260                 265

CGG CGG ACA GCG GCT GGA TCA GCA GTG AGC ATC TGC CAG CAC TCA AGA     1167
Arg Arg Thr Ala Ala Gly Ser Ala Val Ser Ile Cys Gln His Ser Arg
    270                 275                 280

AGG ACA CAA TAT TTC TAT AGT TGG CTA CTA AAT GTG CTC TTA GGC TTA     1215
Arg Thr Gln Tyr Phe Tyr Ser Trp Leu Leu Asn Val Leu Leu Gly Leu
285                 290                 295                 300

CTC GTT CCT GTC GAG GAT GAA CAC TCC ACT CTG CTG ATT CTT GGC GTG     1263
Leu Val Pro Val Glu Asp Glu His Ser Thr Leu Leu Ile Leu Gly Val
                305                 310                 315

CTG CTC ACC CTG AGG TAT TTG GTG CCC TTG CTG CAG CAG CAG GTC AAG     1311
Leu Leu Thr Leu Arg Tyr Leu Val Pro Leu Leu Gln Gln Gln Val Lys
            320                 325                 330

GAC ACA AGC CTG AAA GGC AGC TTC GGA GTG ACA AGG AAA GAA ATG GAA     1359
Asp Thr Ser Leu Lys Gly Ser Phe Gly Val Thr Arg Lys Glu Met Glu
        335                 340                 345

GTC TCT CCT TCT GCA GAG CAG CTT GTC CAG GTT TAT GAA CTG ACG TTA     1407
Val Ser Pro Ser Ala Glu Gln Leu Val Gln Val Tyr Glu Leu Thr Leu
    350                 355                 360

CAT CAT ACA CAG CAC CAA GAC CAC AAT GTT GTG ACC GGA GCC CTG GAG     1455
His His Thr Gln His Gln Asp His Asn Val Val Thr Gly Ala Leu Glu
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                365                 370                 375                 380

CTG TTG CAG CAG CTC TTC AGA ACG CCT CCA CCC GAG CTT CTG CAA ACC          1503
Leu Leu Gln Gln Leu Phe Arg Thr Pro Pro Pro Glu Leu Leu Gln Thr
                385                 390                 395

CTG ACC GCA GTC GGG GGC ATT GGG CAG CTC ACC GCT GCT AAG GAG GAG          1551
Leu Thr Ala Val Gly Gly Ile Gly Gln Leu Thr Ala Ala Lys Glu Glu
            400                 405                 410

TCT GGT GGC CGA AGC CGT AGT GGG AGT ATT GTG GAA CTT ATA GCT GGA          1599
Ser Gly Gly Arg Ser Arg Ser Gly Ser Ile Val Glu Leu Ile Ala Gly
        415                 420                 425

GGG GGT TCC TCA TGC AGC CCT GTC CTT TCA AGA AAA CAA AAA GGC AAA          1647
Gly Gly Ser Ser Cys Ser Pro Val Leu Ser Arg Lys Gln Lys Gly Lys
    430                 435                 440

GTG CTC TTA GGA GAA GAA GAA GCC TTG GAG GAT GAC TCT GAA TCG AGA          1695
Val Leu Leu Gly Glu Glu Glu Ala Leu Glu Asp Asp Ser Glu Ser Arg
445                 450                 455                 460

TCG GAT GTC AGC AGC TCT GCC TTA ACA GCC TCA GTG AAG GAT GAG ATC          1743
Ser Asp Val Ser Ser Ser Ala Leu Thr Ala Ser Val Lys Asp Glu Ile
                465                 470                 475

AGT GGA GAG CTG GCT GCT TCT TCA GGG GTT TCC ACT CCA GGG TCA GCA          1791
Ser Gly Glu Leu Ala Ala Ser Ser Gly Val Ser Thr Pro Gly Ser Ala
            480                 485                 490

GGT CAT GAC ATC ATC ACA GAA CAG CCA CGG TCA CAG CAC ACA CTG CAG          1839
Gly His Asp Ile Ile Thr Glu Gln Pro Arg Ser Gln His Thr Leu Gln
        495                 500                 505

GCG GAC TCA CTG GAT CTG GCC AGC TGT GAC TTG ACA AGC TCT GCC ACT          1887
Ala Asp Ser Leu Asp Leu Ala Ser Cys Asp Leu Thr Ser Ser Ala Thr
    510                 515                 520

GAT GGG GAT GAG GAG GAT ATC TTG AGC CAC AGC TCC AGC CAG GTC AGC          1935
Asp Gly Asp Glu Glu Asp Ile Leu Ser His Ser Ser Ser Gln Val Ser
525                 530                 535                 540

GCC GTC CCA TCT GAC CCT GCC ATG GAC CTG AAT GAT GGG ACC CAG GCC          1983
Ala Val Pro Ser Asp Pro Ala Met Asp Leu Asn Asp Gly Thr Gln Ala
                545                 550                 555

TCG TCG CCC ATC AGC GAC AGC TCC CAG ACC ACC ACC GAA GGG CCT GAT          2031
Ser Ser Pro Ile Ser Asp Ser Ser Gln Thr Thr Thr Glu Gly Pro Asp
            560                 565                 570

TCA GCT GTT ACC CCT TCA GAC AGT TCT GAA ATT GTG TTA GAC GGT ACC          2079
Ser Ala Val Thr Pro Ser Asp Ser Ser Glu Ile Val Leu Asp Gly Thr
        575                 580                 585

GAC AAC CAG TAT TTG GGC CTG CAG ATT GGA CAG CCC CAG GAT GAA GAT          2127
Asp Asn Gln Tyr Leu Gly Leu Gln Ile Gly Gln Pro Gln Asp Glu Asp
    590                 595                 600

GAG GAA GCC ACA GGT ATT CTT CCT CAT CAA GCC TCG CAC CCC TTC ACG          2175
Glu Glu Ala Thr Gly Ile Leu Pro Asp Glu Ala Ser Glu Ala Phe Arg
605                 610                 615                 620

AAC TCT TCC ATG GCC CTT CAA CAG GCA CAT TTA TTG AAA AAC ATG AGT          2223
Asn Ser Ser Met Ala Leu Gln Gln Ala His Leu Leu Lys Asn Met Ser
                625                 630                 635

CAC TGC AGG CAG CCT TCT GAC AGC AGT GTT GAT AAA TTT GTG TTG AGA          2271
His Cys Arg Gln Pro Ser Asp Ser Ser Val Asp Lys Phe Val Leu Arg
            640                 645                 650

GAT GAA GCT ACT GAA CCG GGT GAT CAA GAA AAC AAG CCT TGC CGC ATC          2319
Asp Glu Ala Thr Glu Pro Gly Asp Gln Glu Asn Lys Pro Cys Arg Ile
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                655                 660                 665

AAA GGT GAC ATT GGA CAG TCC ACT GAT GAT GAC TCT GCA CCT CTT GTC      2367
Lys Gly Asp Ile Gly Gln Ser Thr Asp Asp Asp Ser Ala Pro Leu Val
    670                 675                 680

CAT TCT GTC CGC CTT TTA TCT GCT TCG TTT TTG CTA ACA GGG GGA AAA      2415
His Ser Val Arg Leu Leu Ser Ala Ser Phe Leu Leu Thr Gly Gly Lys
685                 690                 695                 700

AAT GTG CTG GTT CCG GAC AGG GAT GTG AGG GTC AGC GTG AAG GCC CTG      2463
Asn Val Leu Val Pro Asp Arg Asp Val Arg Val Ser Val Lys Ala Leu
                705                 710                 715

GCC CTC AGC TGT GTG GGA GCA GCT GTG GCC CTC CAC CCG GAA TCT TTC      2511
Ala Leu Ser Cys Val Gly Ala Ala Val Ala Leu His Pro Glu Ser Phe
            720                 725                 730

TTC AGC AAA CTC TAT AAA GTT CCT CTT GAC ACC ACG GAA TAC CCT GAG      2559
Phe Ser Lys Leu Tyr Lys Val Pro Leu Asp Thr Thr Glu Tyr Pro Glu
        735                 740                 745

GAA CAG TAT GTC TCA GAC ATC TTG AAC TAC ATC GAT CAT GGA GAC CCA      2607
Glu Gln Tyr Val Ser Asp Ile Leu Asn Tyr Ile Asp His Gly Asp Pro
    750                 755                 760

CAG GTT CGA GGA GCC ACT GCC ATT CTC TGT GGG ACC CTC ATC TGC TCC      2655
Gln Val Arg Gly Ala Thr Ala Ile Leu Cys Gly Thr Leu Ile Cys Ser
765                 770                 775                 780

ATC CTC AGC AGG TCC CGC TTC CAC GTG GGA GAT TGG ATG GGC ACC ATT      2703
Ile Leu Ser Arg Ser Arg Phe His Val Gly Asp Trp Met Gly Thr Ile
                785                 790                 795

AGA ACC CTC ACA GGA AAT ACA TTT TCT TTG GCG GAT TGC ATT CCT TTG      2751
Arg Thr Leu Thr Gly Asn Thr Phe Ser Leu Ala Asp Cys Ile Pro Leu
            800                 805                 810

CTG CGG AAA ACA CTG AAG GAT GAG TCT TCT GTT ACT TGC AAG TTA GCT      2799
Leu Arg Lys Thr Leu Lys Asp Glu Ser Ser Val Thr Cys Lys Leu Ala
        815                 820                 825

TGT ACA GCT GTG AGG AAC TGT GTC ATG AGT CTC TGC AGC AGC AGC TAC      2847
Cys Thr Ala Val Arg Asn Cys Val Met Ser Leu Cys Ser Ser Ser Tyr
    830                 835                 840

AGT GAG TTA GGA CTG CAG CTG ATC ATC GAT GTG CTG ACT CTG AGG AAC      2895
Ser Glu Leu Gly Leu Gln Leu Ile Ile Asp Val Leu Thr Leu Arg Asn
845                 850                 855                 860

AGT TCC TAT TGG CTG GTG AGG ACA GAG CTT CTG GAA ACC CTT GCA GAG      2943
Ser Ser Tyr Trp Leu Val Arg Thr Glu Leu Leu Glu Thr Leu Ala Glu
                865                 870                 875

ATT GAC TTC AGG CTG GTG AGC TTT TTG GAG GCA AAA GCA GAA AAC TTA      2991
Ile Asp Phe Arg Leu Val Ser Phe Leu Glu Ala Lys Ala Glu Asn Leu
            880                 885                 890

CAC AGA GGG GCT CAT CAT TAT ACA GGG CTT TTA AAA CTG CAA GAA CGA      3039
His Arg Gly Ala His His Tyr Thr Gly Leu Leu Lys Leu Gln Glu Arg
        895                 900                 905

GTG CTC AAT AAT GTT GTC ATC CAT TTG CTT GGA GAT GAA GAC CCC AGG      3087
Val Leu Asn Asn Val Val Ile His Leu Leu Gly Asp Glu Asp Pro Arg
    910                 915                 920

GTG CGA CAT GTT GCC GCA GCA TCA CTA ATT AGG CTT GTC CCA AAG CTG      3135
Val Arg His Val Ala Ala Ala Ser Leu Ile Arg Leu Val Pro Lys Leu
925                 930                 935                 940

TTT TAT AAA TGT GAC CAA GGA CAA GCT GAT CCA GTA GTG GCC GTG GCA      3183
Phe Tyr Lys Cys Asp Gln Gly Gln Ala Asp Pro Val Val Ala Val Ala
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                    945                 950                 955

AGA GAT CAA AGC AGT GTT TAC CTG AAA CTT CTC ATG CAT GAG ACG CAG      3231
Arg Asp Gln Ser Ser Val Tyr Leu Lys Leu Leu Met His Glu Thr Gln
            960                 965                 970

CCT CCA TCT CAT TTC TCC GTC AGC ACA ATA ACC AGA ATA TAT AGA GGC      3279
Pro Pro Ser His Phe Ser Val Ser Thr Ile Thr Arg Ile Tyr Arg Gly
        975                 980                 985

TAT AAC CTA CTA CCA AGC ATA ACA GAC GTC ACT ATG GAA AAT AAC CTT      3327
Tyr Asn Leu Leu Pro Ser Ile Thr Asp Val Thr Met Glu Asn Asn Leu
    990                 995                1000

TCA AGA GTT ATT GCA GCA GTT TCT CAT GAA CTA ATC ACA TCA ACC ACC      3375
Ser Arg Val Ile Ala Ala Val Ser His Glu Leu Ile Thr Ser Thr Thr
1005                1010                1015                1020

AGA GCA CTC ACA TTT GGA TGC TGT GAA GCT TTG TGT CTT CTT TCC ACT      3423
Arg Ala Leu Thr Phe Gly Cys Cys Glu Ala Leu Cys Leu Leu Ser Thr
                1025                1030                1035

GCC TTC CCA GTT TGC ATT TGG AGT TTA GGT TGG CAC TGT GGA GTG CCT      3471
Ala Phe Pro Val Cys Ile Trp Ser Leu Gly Trp His Cys Gly Val Pro
            1040                1045                1050

CCA CTG AGT GCC TCA GAT GAG TCT AGG AAG AGC TGT ACC GTT GGG ATG      3519
Pro Leu Ser Ala Ser Asp Glu Ser Arg Lys Ser Cys Thr Val Gly Met
        1055                1060                1065

GCC ACA ATG ATT CTG ACC CTG CTC TCG TCA GCT TGG TTC CCA TTG GAT      3567
Ala Thr Met Ile Leu Thr Leu Leu Ser Ser Ala Trp Phe Pro Leu Asp
    1070                1075                1080

CTC TCA GCC CAT CAA GAT GCT TTG ATT TTG GCC GGA AAC TTG CTT GCA      3615
Leu Ser Ala His Gln Asp Ala Leu Ile Leu Ala Gly Asn Leu Leu Ala
1085                1090                1095                1100

GCC AGT GCT CCC AAA TCT CTG AGA AGT TCA TGG GCC TCT GAA GAA GAA      3663
Ala Ser Ala Pro Lys Ser Leu Arg Ser Ser Trp Ala Ser Glu Glu Glu
                1105                1110                1115

GCC AAC CCA GCA GCC ACC AAG CAA GAG GAG GTC TGG CCA GCC CTG GGG      3711
Ala Asn Pro Ala Ala Thr Lys Gln Glu Glu Val Trp Pro Ala Leu Gly
            1120                1125                1130

GAC CGG GCC CTG GTG CCC ATG GTG GAG CAG CTC TTC TCT CAC CTG CTG      3759
Asp Arg Ala Leu Val Pro Met Val Glu Gln Leu Phe Ser His Leu Leu
        1135                1140                1145

AAG GTG ATT AAC ATT TGT GCC CAC GTC CTG GAT GAC GTG GCT CCT GGA      3807
Lys Val Ile Asn Ile Cys Ala His Val Leu Asp Asp Val Ala Pro Gly
    1150                1155                1160

CCC GCA ATA AAG GCA GCC TTG CCT TCT CTA ACA AAC CCC CCT TCT CTA      3855
Pro Ala Ile Lys Ala Ala Leu Pro Ser Leu Thr Asn Pro Pro Ser Leu
1165                1170                1175                1180

AGT CCC ATC CGA CGA AAG GGG AAG GAG AAA GAA CCA GGA GAA CAA GCA      3903
Ser Pro Ile Arg Arg Lys Gly Lys Glu Lys Glu Pro Gly Glu Gln Ala
                1185                1190                1195

TCT GTA CCG TTG AGT CCC AAG AAA GGC AGT GAG GCC AGT GCA GCT TCT      3951
Ser Val Pro Leu Ser Pro Lys Lys Gly Ser Glu Ala Ser Ala Ala Ser
            1200                1205                1210

AGA CAA TCT GAT ACC TCA GGT CCT GTT ACA ACA AGT AAA TCC TCA TCA      3999
Arg Gln Ser Asp Thr Ser Gly Pro Val Thr Thr Ser Lys Ser Ser Ser
        1215                1220                1225

CTG GGG AGT TTC TAT CAT CTT CCT TCA TAC CTC AGA CTG CAT GAT GTC      4047
Leu Gly Ser Phe Tyr His Leu Pro Ser Tyr Leu Arg Leu His Asp Val
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    1230                1235                1240

CTG AAA GCT ACA CAC GCT AAC TAC AAG GTC ACG CTG GAT CTT CAG AAC      4095
Leu Lys Ala Thr His Ala Asn Tyr Lys Val Thr Leu Asp Leu Gln Asn
1245                1250                1255                1260

AGC ACG GAA AAG TTT GGA GGG TTT CTC CGC TCA GCC TTG GAT GTT CTT      4143
Ser Thr Glu Lys Phe Gly Gly Phe Leu Arg Ser Ala Leu Asp Val Leu
                1265                1270                1275

TCT CAG ATA CTA GAG CTG GCC ACA CTG CAG GAC ATT GGG AAG TGT GTT      4191
Ser Gln Ile Leu Glu Leu Ala Thr Leu Gln Asp Ile Gly Lys Cys Val
            1280                1285                1290

GAA GAG ATC CTA GGA TAC CTG AAA TCC TGC TTT AGT CGA GAA CCA ATG      4239
Glu Glu Ile Leu Gly Tyr Leu Lys Ser Cys Phe Ser Arg Glu Pro Met
        1295                1300                1305

ATG GCA ACT GTT TGT GTT CAA CAA TTG TTG AAG ACT CTC TTT GGC ACA      4287
Met Ala Thr Val Cys Val Gln Gln Leu Leu Lys Thr Leu Phe Gly Thr
    1310                1315                1320

AAC TTG GCC TCC CAG TTT GAT GGC TTA TCT TCC AAC CCC AGC AAG TCA      4335
Asn Leu Ala Ser Gln Phe Asp Gly Leu Ser Ser Asn Pro Ser Lys Ser
1325                1330                1335                1340

CAA GGC CGA GCA CAG CGC CTT GGC TCC TCC AGT GTG AGG CCA GGC TTG      4383
Gln Gly Arg Ala Gln Arg Leu Gly Ser Ser Ser Val Arg Pro Gly Leu
                1345                1350                1355

TAC CAC TAC TGC TTC ATG GCC CCG TAC ACC CAC TTC ACC CAG GCC CTC      4431
Tyr His Tyr Cys Phe Met Ala Pro Tyr Thr His Phe Thr Gln Ala Leu
            1360                1365                1370

GCT GAC GCC AGC CTG AGG AAC ATG GTG CAG GCG GAG CAG GAG AAC GAC      4479
Ala Asp Ala Ser Leu Arg Asn Met Val Gln Ala Glu Gln Glu Asn Asp
        1375                1380                1385

ACC TCG GGA TGG TTT GAT GTC CTC CAG AAA GTG TCT ACC CAG TTG AAG      4527
Thr Ser Gly Trp Phe Asp Val Leu Gln Lys Val Ser Thr Gln Leu Lys
    1390                1395                1400

ACA AAC CTC ACG AGT GTC ACA AAG AAC CGT GCA GAT AAG AAT GCT ATT      4575
Thr Asn Leu Thr Ser Val Thr Lys Asn Arg Ala Asp Lys Asn Ala Ile
1405                1410                1415                1420

CAT AAT CAC ATT CGT TTG TTT GAA CCT CTT GTT ATA AAA GCT TTA AAA      4623
His Asn His Ile Arg Leu Phe Glu Pro Leu Val Ile Lys Ala Leu Lys
                1425                1430                1435

CAG TAC ACG ACT ACA ACA TGT GTG CAG TTA CAG AAG CAG GTT TTA GAT      4671
Gln Tyr Thr Thr Thr Thr Cys Val Gln Leu Gln Lys Gln Val Leu Asp
            1440                1445                1450

TTG CTG GCG CAG CTG GTT CAG TTA CGG GTT AAT TAC TGT CTT CTG GAT      4719
Leu Leu Ala Gln Leu Val Gln Leu Arg Val Asn Tyr Cys Leu Leu Asp
        1455                1460                1465

TCA GAT CAG GTG TTT ATT GGC TTT GTA TTG AAA CAG TTT GAA TAC ATT      4767
Ser Asp Gln Val Phe Ile Gly Phe Val Leu Lys Gln Phe Glu Tyr Ile
    1470                1475                1480

GAA GTG GGC CAG TTC AGG GAA TCA GAG GCA ATC ATT CCA AAC ATC TTT      4815
Glu Val Gly Gln Phe Arg Glu Ser Glu Ala Ile Ile Pro Asn Ile Phe
1485                1490                1495                1500

TTC TTC TTG GTA TTA CTA TCT TAT GAA CGC TAT CAT TCA AAA CAG ATC      4863
Phe Phe Leu Val Leu Leu Ser Tyr Glu Arg Tyr His Ser Lys Gln Ile
                1505                1510                1515

ATT GGA ATT CCT AAA ATC ATT CAG CTC TGT GAT GGC ATC ATG GCC AGT      4911
Ile Gly Ile Pro Lys Ile Ile Gln Leu Cys Asp Gly Ile Met Ala Ser
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                1520                1525                1530

GGA AGG AAG GCT GTG ACA CAT GCC ATA CCG GCT CTG CAG CCC ATA GTC      4959
Gly Arg Lys Ala Val Thr His Ala Ile Pro Ala Leu Gln Pro Ile Val
        1535                1540                1545

CAC GAC CTC TTT GTA TTA AGA GGA ACA AAT AAA GCT GAT GCA GGA AAA      5007
His Asp Leu Phe Val Leu Arg Gly Thr Asn Lys Ala Asp Ala Gly Lys
    1550                1555                1560

GAG CTT GAA ACC CAA AAA GAG GTG GTG GTG TCA ATG TTA CTG AGA CTC      5055
Glu Leu Glu Thr Gln Lys Glu Val Val Val Ser Met Leu Leu Arg Leu
1565                1570                1575                1580

ATC CAG TAC CAT CAG GTG TTG GAG ATG TTC ATT CTT GTC CTG CAG CAG      5103
Ile Gln Tyr His Gln Val Leu Glu Met Phe Ile Leu Val Leu Gln Gln
                1585                1590                1595

TGC CAC AAG GAG AAT GAA GAC AAG TGG AAG CGA CTG TCT CGA CAG ATA      5151
Cys His Lys Glu Asn Glu Asp Lys Trp Lys Arg Leu Ser Arg Gln Ile
            1600                1605                1610

GCT GAC ATC ATC CTC CCA ATG TTA GCC AAA CAG CAG ATG CAC ATT GAC      5199
Ala Asp Ile Ile Leu Pro Met Leu Ala Lys Gln Gln Met His Ile Asp
        1615                1620                1625

TCT CAT GAA GCC CTT GGA GTG TTA AAT ACA TTA TTT GAG ATT TTG GCC      5247
Ser His Glu Ala Leu Gly Val Leu Asn Thr Leu Phe Glu Ile Leu Ala
    1630                1635                1640

CCT TCC TCC CTC CGT CCG GTA GAC ATG CTT TTA CGG AGT ATG TTC GTC      5295
Pro Ser Ser Leu Arg Pro Val Asp Met Leu Leu Arg Ser Met Phe Val
1645                1650                1655                1660

ACT CCA AAC ACA ATG GCG TCC GTG AGC ACT GTT CAA CTG TGG ATA TCG      5343
Thr Pro Asn Thr Met Ala Ser Val Ser Thr Val Gln Leu Trp Ile Ser
                1665                1670                1675

GGA ATT CTG GCC ATT TTG AGG GTT CTG ATT TCC CAG TCA ACT GAA GAT      5391
Gly Ile Leu Ala Ile Leu Arg Val Leu Ile Ser Gln Ser Thr Glu Asp
            1680                1685                1690

ATT GTT CTT TCT CGT ATT CAG GAG CTC TCC TTC TCT CCG TAT TTA ATC      5439
Ile Val Leu Ser Arg Ile Gln Glu Leu Ser Phe Ser Pro Tyr Leu Ile
        1695                1700                1705

TCC TGT ACA GTA ATT AAT AGG TTA AGA GAT GGG GAC AGT ACT TCA ACG      5487
Ser Cys Thr Val Ile Asn Arg Leu Arg Asp Gly Asp Ser Thr Ser Thr
    1710                1715                1720

CTA GAA GAA CAC AGT GAA GGG AAA CAA ATA AAG AAT TTG CCA GAA GAA      5535
Leu Glu Glu His Ser Glu Gly Lys Gln Ile Lys Asn Leu Pro Glu Glu
1725                1730                1735                1740

ACA TTT TCA AGG TTT CTA TTA CAA CTG GTT GGT ATT CTT TTA GAA GAC      5583
Thr Phe Ser Arg Phe Leu Leu Gln Leu Val Gly Ile Leu Leu Glu Asp
                1745                1750                1755

ATT GTT ACA AAA CAG CTG AAG GTG GAA ATG AGT GAG CAG CAA CAT ACT      5631
Ile Val Thr Lys Gln Leu Lys Val Glu Met Ser Glu Gln Gln His Thr
            1760                1765                1770

TTC TAT TGC CAG GAA CTA GGC ACA CTG CTA ATG TGT CTG ATC CAC ATC      5679
Phe Tyr Cys Gln Glu Leu Gly Thr Leu Leu Met Cys Leu Ile His Ile
        1775                1780                1785

TTC AAG TCT GGA ATG TTC CGG AGA ATC ACA GCA GCT GCC ACT AGG CTG      5727
Phe Lys Ser Gly Met Phe Arg Arg Ile Thr Ala Ala Ala Thr Arg Leu
    1790                1795                1800

TTC CGC AGT GAT GGC TGT GGC GGC AGT TTC TAC ACC CTG GAC AGC TTG      5775
Phe Arg Ser Asp Gly Cys Gly Gly Ser Phe Tyr Thr Leu Asp Ser Leu
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1805                1810                1815                1820

AAC TTG CGG GCT CGT TCC ATG ATC ACC ACC CAC CCG GCC CTG GTG CTG      5823
Asn Leu Arg Ala Arg Ser Met Ile Thr Thr His Pro Ala Leu Val Leu
                1825                1830                1835

CTC TGG TGT CAG ATA CTG CTG CTT GTC AAC CAC ACC GAC TAC CGC TGG      5871
Leu Trp Cys Gln Ile Leu Leu Leu Val Asn His Thr Asp Tyr Arg Trp
            1840                1845                1850

TGG GCA GAA GTG CAG CAG ACC CCG AAA AGA CAC AGT CTG TCC AGC ACA      5919
Trp Ala Glu Val Gln Gln Thr Pro Lys Arg His Ser Leu Ser Ser Thr
        1855                1860                1865

AAG TTA CTT AGT CCC CAG ATG TCT GGA GAA GAG GAG GAT TCT GAC TTG      5967
Lys Leu Leu Ser Pro Gln Met Ser Gly Glu Glu Glu Asp Ser Asp Leu
    1870                1875                1880

GCA GCC AAA CTT GGA ATG TGC AAT AGA GAA ATA GTA CGA AGA GGG GCT      6015
Ala Ala Lys Leu Gly Met Cys Asn Arg Glu Ile Val Arg Arg Gly Ala
1885                1890                1895                1900

CTC ATT CTC TTC TGT GAT TAT GTC TGT CAG AAC CTC CAT GAC TCC GAG      6063
Leu Ile Leu Phe Cys Asp Tyr Val Cys Gln Asn Leu His Asp Ser Glu
                1905                1910                1915

CAC TTA ACG TGG CTC ATT GTA AAT CAC ATT CAA GAT CTG ATC AGC CTT      6111
His Leu Thr Trp Leu Ile Val Asn His Ile Gln Asp Leu Ile Ser Leu
            1920                1925                1930

TCC CAC GAG CCT CCA GTA CAG GAC TTC ATC AGT GCC GTT CAT CGG AAC      6159
Ser His Glu Pro Pro Val Gln Asp Phe Ile Ser Ala Val His Arg Asn
        1935                1940                1945

TCT GCT GCC AGC GGC CTG TTC ATC CAG GCA ATT CAG TCT CGT TGT GAA      6207
Ser Ala Ala Ser Gly Leu Phe Ile Gln Ala Ile Gln Ser Arg Cys Glu
    1950                1955                1960

AAC CTT TCA ACT CCA ACC ATG CTG AAG AAA ACT CTT CAG TGC TTG GAG      6255
Asn Leu Ser Thr Pro Thr Met Leu Lys Lys Thr Leu Gln Cys Leu Glu
1965                1970                1975                1980

GGG ATC CAT CTC AGC CAG TCG GGA GCT GTG CTC ACG CTG TAT GTG GAC      6303
Gly Ile His Leu Ser Gln Ser Gly Ala Val Leu Thr Leu Tyr Val Asp
                1985                1990                1995

AGG CTT CTG TGC ACC CCT TTC CGT GTG CTG GCT CGC ATG GTC GAC ATC      6351
Arg Leu Leu Cys Thr Pro Phe Arg Val Leu Ala Arg Met Val Asp Ile
            2000                2005                2010

CTT GCT TGT CGC CGG GTA GAA ATG CTT CTG GCT GCA AAT TTA CAG AGC      6399
Leu Ala Cys Arg Arg Val Glu Met Leu Leu Ala Ala Asn Leu Gln Ser
        2015                2020                2025

AGC ATG GCC CAG TTG CCA ATG GAA GAA CTC AAC AGA ATC CAG GAA TAC      6447
Ser Met Ala Gln Leu Pro Met Glu Glu Leu Asn Arg Ile Gln Glu Tyr
    2030                2035                2040

CTT CAG AGC AGC GGG CTC GCT CAG AGA CAC CAA AGG CTC TAT TCC CTG      6495
Leu Gln Ser Ser Gly Leu Ala Gln Arg His Gln Arg Leu Tyr Ser Leu
2045                2050                2055                2060

CTG GAC AGG TTT CGT CTC TCC ACC ATG CAA GAC TCA CTT AGT CCC TCT      6543
Leu Asp Arg Phe Arg Leu Ser Thr Met Gln Asp Ser Leu Ser Pro Ser
                2065                2070                2075

CCT CCA GTC TCT TCC CAC CCG CTG GAC GGG GAT GGG CAC GTG TCA CTG      6591
Pro Pro Val Ser Ser His Pro Leu Asp Gly Asp Gly His Val Ser Leu
            2080                2085                2090

GAA ACA GTG AGT CCG GAC AAA GAC TGG TAC GTT CAT CTT GTC AAA TCC      6639
Glu Thr Val Ser Pro Asp Lys Asp Trp Tyr Val His Leu Val Lys Ser
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          2095                2100                2105

CAG TGT TGG ACC AGG TCA GAT TCT GCA CTG CTG GAA GGT GCA GAG CTG      6687
Gln Cys Trp Thr Arg Ser Asp Ser Ala Leu Leu Glu Gly Ala Glu Leu
     2110                2115                2120

GTG AAT CGG ATT CCT GCT GAA GAT ATG AAT GCC TTC ATG ATG AAC TCG      6735
Val Asn Arg Ile Pro Ala Glu Asp Met Asn Ala Phe Met Met Asn Ser
2125                2130                2135                2140

GAG TTC AAC CTA AGC CTG CTA GCT CCA TGC TTA AGC CTA GGG ATG AGT      6783
Glu Phe Asn Leu Ser Leu Leu Ala Pro Cys Leu Ser Leu Gly Met Ser
                2145                2150                2155

GAA ATT TCT GGT GGC CAG AAG AGT GCC CTT TTT GAA GCA GCC CGT GAG      6831
Glu Ile Ser Gly Gly Gln Lys Ser Ala Leu Phe Glu Ala Ala Arg Glu
          2160                2165                2170

GTG ACT CTG GCC CGT GTG AGC GGC ACC GTG CAG CAG CTC CCT GCT GTC      6879
Val Thr Leu Ala Arg Val Ser Gly Thr Val Gln Gln Leu Pro Ala Val
     2175                2180                2185

CAT CAT GTC TTC CAG CCC GAG CTG CCT GCA GAG CCG GCG GCC TAC TGG      6927
His His Val Phe Gln Pro Glu Leu Pro Ala Glu Pro Ala Ala Tyr Trp
2190                2195                2200

AGC AAG TTG AAT GAT CTG TTT GGG GAT GCT GCA CTG TAT CAG TCC CTG      6975
Ser Lys Leu Asn Asp Leu Phe Gly Asp Ala Ala Leu Tyr Gln Ser Leu
2205                2210                2215                2220

CCC ACT CTG GCC CGG GCC CTG GCA CAG TAC CTG GTG GTG GTC TCC AAA      7023
Pro Thr Leu Ala Arg Ala Leu Ala Gln Tyr Leu Val Val Val Ser Lys
                2225                2230                2235

CTG CCC AGT CAT TTG CAC CTT CCT CCT GAG AAA GAG AAG GAC ATT GTG      7071
Leu Pro Ser His Leu His Leu Pro Pro Glu Lys Glu Lys Asp Ile Val
          2240                2245                2250

AAA TTC GTG GTG GCA ACC CTT GAG GCC CTG TCC TGG CAT TTG ATC CAT      7119
Lys Phe Val Val Ala Thr Leu Glu Ala Leu Ser Trp His Leu Ile His
     2255                2260                2265

GAG CAG ATC CCG CTG AGT CTG GAT CTC CAG GCA GGG CTG GAC TGC TGC      7167
Glu Gln Ile Pro Leu Ser Leu Asp Leu Gln Ala Gly Leu Asp Cys Cys
2270                2275                2280

TGC CTG GCC CTG CAG CTG CCT GGC CTC TGG AGC GTG GTC TCC TCC ACA      7215
Cys Leu Ala Leu Gln Leu Pro Gly Leu Trp Ser Val Val Ser Ser Thr
2285                2290                2295                2300

GAG TTT GTG ACC CAC GCC TGC TCC CTC ATC TAC TGT GTG CAC TTC ATC      7263
Glu Phe Val Thr His Ala Cys Ser Leu Ile Tyr Cys Val His Phe Ile
                2305                2310                2315

CTG GAG GCC GTT GCA GTG CAG CCT GGA GAG CAG CTT CTT AGT CCA GAA      7311
Leu Glu Ala Val Ala Val Gln Pro Gly Glu Gln Leu Leu Ser Pro Glu
          2320                2325                2330

AGA AGG ACA AAT ACC CCA AAA GCC ATC AGC GAG GAG GAG GAG GAA GTA      7359
Arg Arg Thr Asn Thr Pro Lys Ala Ile Ser Glu Glu Glu Glu Glu Val
     2335                2340                2345

GAT CCA AAC ACA CAG AAT CCT AAG TAT ATC ACT GCA GCC TGT GAG ATG      7407
Asp Pro Asn Thr Gln Asn Pro Lys Tyr Ile Thr Ala Ala Cys Glu Met
2350                2355                2360

GTG GCA GAA ATG GTG GAG TCT CTG CAG TCG GTG TTG GCC TTG GGT CAT      7455
Val Ala Glu Met Val Glu Ser Leu Gln Ser Val Leu Ala Leu Gly His
2365                2370                2375                2380

AAA AGG AAT AGC GGC GTG CCG GCG TTT CTC ACG CCA TTG CTC AGG AAC      7503
Lys Arg Asn Ser Gly Val Pro Ala Phe Leu Thr Pro Leu Leu Arg Asn
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                    2385                2390                2395

ATC ATC ATC AGC CTG GCC CGC CTG CCC CTT GTC AAC AGC TAC ACA CGT      7551
Ile Ile Ile Ser Leu Ala Arg Leu Pro Leu Val Asn Ser Tyr Thr Arg
            2400                2405                2410

GTG CCC CCA CTG GTG TGG AAG CTT GGA TGG TCA CCC AAA CCG GGA GGG      7599
Val Pro Pro Leu Val Trp Lys Leu Gly Trp Ser Pro Lys Pro Gly Gly
        2415                2420                2425

GAT TTT GGC ACA GCA TTC CCT GAG ATC CCC GTG GAG TTC CTC CAG GAA      7647
Asp Phe Gly Thr Ala Phe Pro Glu Ile Pro Val Glu Phe Leu Gln Glu
    2430                2435                2440

AAG GAA GTC TTT AAG GAG TTC ATC TAC CGC ATC AAC ACA CTA GGC TGG      7695
Lys Glu Val Phe Lys Glu Phe Ile Tyr Arg Ile Asn Thr Leu Gly Trp
2445                2450                2455                2460

ACC AGT CGT ACT CAG TTT GAA GAA ACT TGG GCC ACC CTC CTT GGT GTC      7743
Thr Ser Arg Thr Gln Phe Glu Glu Thr Trp Ala Thr Leu Leu Gly Val
                2465                2470                2475

CTG GTG ACG CAG CCC CTC GTG ATG GAG CAG GAG GAG AGC CCA CCA GAA      7791
Leu Val Thr Gln Pro Leu Val Met Glu Gln Glu Glu Ser Pro Pro Glu
            2480                2485                2490

GAA GAC ACA GAG AGG ACC CAG ATC AAC GTC CTG GCC GTG CAG GCC ATC      7839
Glu Asp Thr Glu Arg Thr Gln Ile Asn Val Leu Ala Val Gln Ala Ile
        2495                2500                2505

ACC TCA CTG GTG CTC AGT GCA ATG ACT GTG CCT GTG GCC GGC AAC CCA      7887
Thr Ser Leu Val Leu Ser Ala Met Thr Val Pro Val Ala Gly Asn Pro
    2510                2515                2520

GCT GTA AGC TGC TTG GAG CAG CAG CCC CGG AAC AAG CCT CTG AAA GCT      7935
Ala Val Ser Cys Leu Glu Gln Gln Pro Arg Asn Lys Pro Leu Lys Ala
2525                2530                2535                2540

CTC GAC ACC AGG TTT GGG AGG AAG CTG AGC ATT ATC AGA GGG ATT GTG      7983
Leu Asp Thr Arg Phe Gly Arg Lys Leu Ser Ile Ile Arg Gly Ile Val
                2545                2550                2555

GAG CAA GAG ATT CAA GCA ATG GTT TCA AAG AGA GAG AAT ATT GCC ACC      8031
Glu Gln Glu Ile Gln Ala Met Val Ser Lys Arg Glu Asn Ile Ala Thr
            2560                2565                2570

CAT CAT TTA TAT CAG GCA TGG GAT CCT GTC CCT TCT CTG TCT CCG GCT      8079
His His Leu Tyr Gln Ala Trp Asp Pro Val Pro Ser Leu Ser Pro Ala
        2575                2580                2585

ACT ACA GGT GCC CTC ATC AGC CAC GAG AAG CTG CTG CTA CAG ATC AAC      8127
Thr Thr Gly Ala Leu Ile Ser His Glu Lys Leu Leu Leu Gln Ile Asn
    2590                2595                2600

CCC GAG CGG GAG CTG GGG AGC ATG AGC TAC AAA CTC GGC CAG GTG TCC      8175
Pro Glu Arg Glu Leu Gly Ser Met Ser Tyr Lys Leu Gly Gln Val Ser
2605                2610                2615                2620

ATA CAC TCC GTG TGG CTG GGG AAC AGC ATC ACA CCC CTG AGG GAG GAG      8223
Ile His Ser Val Trp Leu Gly Asn Ser Ile Thr Pro Leu Arg Glu Glu
                2625                2630                2635

GAA TGG GAC GAG GAA GAG GAG GAG GAG GCC GAC GCC CCT GCA CCT TCG      8271
Glu Trp Asp Glu Glu Glu Glu Glu Glu Ala Asp Ala Pro Ala Pro Ser
            2640                2645                2650

TCA CCA CCC ACG TCT CCA GTC AAC TCC AGG AAA CAC CGG GCT GGA GTT      8319
Ser Pro Pro Thr Ser Pro Val Asn Ser Arg Lys His Arg Ala Gly Val
        2655                2660                2665

GAC ATC CAC TCC TGT TCG CAG TTT TTG CTT GAG TTG TAC AGC CGC TGG      8367
Asp Ile His Ser Cys Ser Gln Phe Leu Leu Glu Leu Tyr Ser Arg Trp
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     2670                2675                2680

ATC CTG CCG TCC AGC TCA GCC AGG AGG ACC CCG GCC ATC CTG ATC AGT      8415
Ile Leu Pro Ser Ser Ser Ala Arg Arg Thr Pro Ala Ile Leu Ile Ser
2685                2690                2695                2700

GAG GTG GTC AGA TCC CTT CTA GTG GTC TCA GAC TTG TTC ACC GAG CGC      8463
Glu Val Val Arg Ser Leu Leu Val Val Ser Asp Leu Phe Thr Glu Arg
                2705                2710                2715

AAC CAG TTT GAG CTG ATG TAT GTG ACG CTG ACA GAA CTG CGA AGG GTG      8511
Asn Gln Phe Glu Leu Met Tyr Val Thr Leu Thr Glu Leu Arg Arg Val
            2720                2725                2730

CAC CCT TCA GAA GAC GAG ATC CTC GCT CAG TAC CTG GTG CCT GCC ACC      8559
His Pro Ser Glu Asp Glu Ile Leu Ala Gln Tyr Leu Val Pro Ala Thr
        2735                2740                2745

TGC AAG GCA GCT GCC GTC CTT GGG ATG GAC AAG GCC GTG GCG GAG CCT      8607
Cys Lys Ala Ala Ala Val Leu Gly Met Asp Lys Ala Val Ala Glu Pro
    2750                2755                2760

GTC AGC CGC CTG CTG GAG AGC ACG CTC AGG AGC AGC CAC CTG CCC AGC      8655
Val Ser Arg Leu Leu Glu Ser Thr Leu Arg Ser Ser His Leu Pro Ser
2765                2770                2775                2780

AGG GTT GGA GCC CTG CAC GGC ATC CTC TAT GTG CTG GAG TGC GAC CTG      8703
Arg Val Gly Ala Leu His Gly Ile Leu Tyr Val Leu Glu Cys Asp Leu
                2785                2790                2795

CTG GAC GAC ACT GCC AAG CAG CTC ATC CCG GTC ATC AGC GAC TAT CTC      8751
Leu Asp Asp Thr Ala Lys Gln Leu Ile Pro Val Ile Ser Asp Tyr Leu
            2800                2805                2810

CTC TCC AAC CTG AAA GGG ATC GCC CAC TGC GTG AAC ATT CAC AGC CAG      8799
Leu Ser Asn Leu Lys Gly Ile Ala His Cys Val Asn Ile His Ser Gln
        2815                2820                2825

CAG CAC GTA CTG GTC ATG TGT GCC ACT GCG TTT TAC CTC ATT GAG AAC      8847
Gln His Val Leu Val Met Cys Ala Thr Ala Phe Tyr Leu Ile Glu Asn
    2830                2835                2840

TAT CCT CTG GAC GTA GGG CCG GAA TTT TCA GCA TCA ATA ATA CAG ATG      8895
Tyr Pro Leu Asp Val Gly Pro Glu Phe Ser Ala Ser Ile Ile Gln Met
2845                2850                2855                2860

TGT GGG GTG ATG CTG TCT GGA AGT GAG GAG TCC ACC CCC TCC ATC ATT      8943
Cys Gly Val Met Leu Ser Gly Ser Glu Glu Ser Thr Pro Ser Ile Ile
                2865                2870                2875

TAC CAC TGT GCC CTC AGA GGC CTG GAG CGC CTC CTG CTC TCT GAG CAG      8991
Tyr His Cys Ala Leu Arg Gly Leu Glu Arg Leu Leu Leu Ser Glu Gln
            2880                2885                2890

CTC TCC CGC CTG GAT GCA GAA TCG CTG GTC AAG CTG AGT GTG GAC AGA      9039
Leu Ser Arg Leu Asp Ala Glu Ser Leu Val Lys Leu Ser Val Asp Arg
        2895                2900                2905

GTG AAC GTG CAC AGC CCG CAC CGG GCC ATG GCG GCT CTG GGC CTG ATG      9087
Val Asn Val His Ser Pro His Arg Ala Met Ala Ala Leu Gly Leu Met
    2910                2915                2920

CTC ACC TGC ATG TAC ACA GGA AAG GAG AAA GTC AGT CCG GGT AGA ACT      9135
Leu Thr Cys Met Tyr Thr Gly Lys Glu Lys Val Ser Pro Gly Arg Thr
2925                2930                2935                2940

TCA GAC CCT AAT CCT GCA GCC CCC GAC AGC GAG TCA GTG ATT GTT GCT      9183
Ser Asp Pro Asn Pro Ala Ala Pro Asp Ser Glu Ser Val Ile Val Ala
                2945                2950                2955

ATG GAG CGG GTA TCT GTT CTT TTT GAT AGG ATC AGG AAA GGC TTT CCT      9231
Met Glu Arg Val Ser Val Leu Phe Asp Arg Ile Arg Lys Gly Phe Pro
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                2960                2965                2970

TGT GAA GCC AGA GTG GTG GCC AGG ATC CTG CCC CAG TTT CTA GAC GAC      9279
Cys Glu Ala Arg Val Val Ala Arg Ile Leu Pro Gln Phe Leu Asp Asp
        2975                2980                2985

TTC TTC CCA CCC CAG GAC ATC ATG AAC AAA GTC ATC GGA GAG TTT CTG      9327
Phe Phe Pro Pro Gln Asp Ile Met Asn Lys Val Ile Gly Glu Phe Leu
    2990                2995                3000

TCC AAC CAG CAG CCA TAC CCC CAG TTC ATG GCC ACC GTG GTG TAT AAG      9375
Ser Asn Gln Gln Pro Tyr Pro Gln Phe Met Ala Thr Val Val Tyr Lys
3005                3010                3015                3020

GTG TTT CAG ACT CTG CAC AGC ACC GGG CAG TCG TCC ATG GTC CGG GAC      9423
Val Phe Gln Thr Leu His Ser Thr Gly Gln Ser Ser Met Val Arg Asp
                3025                3030                3035

TGG GTC ATG CTG TCC CTC TCC AAC TTC ACG CAG AGG GCC CCG GTC GCC      9471
Trp Val Met Leu Ser Leu Ser Asn Phe Thr Gln Arg Ala Pro Val Ala
            3040                3045                3050

ATG GCC ACG TGG AGC CTC TCC TGC TTC TTT GTC AGC GCG TCC ACC AGC      9519
Met Ala Thr Trp Ser Leu Ser Cys Phe Phe Val Ser Ala Ser Thr Ser
        3055                3060                3065

CCG TGG GTC GCG GCG ATC CTC CCA CAT GTC ATC AGC AGG ATG GGC AAG      9567
Pro Trp Val Ala Ala Ile Leu Pro His Val Ile Ser Arg Met Gly Lys
    3070                3075                3080

CTG GAG CAG GTG GAC GTG AAC CTT TTC TGC CTG GTC GCC ACA GAC TTC      9615
Leu Glu Gln Val Asp Val Asn Leu Phe Cys Leu Val Ala Thr Asp Phe
3085                3090                3095                3100

TAC AGA CAC CAG ATA GAG GAG GAG CTC GAC CGC AGG GCC TTC CAG TCT      9663
Tyr Arg His Gln Ile Glu Glu Glu Leu Asp Arg Arg Ala Phe Gln Ser
                3105                3110                3115

GTG CTT GAG GTG GTT GCA GCC CCA GGA AGC CCA TAT CAC CGG CTG CTG      9711
Val Leu Glu Val Val Ala Ala Pro Gly Ser Pro Tyr His Arg Leu Leu
            3120                3125                3130

ACT TGT TTA CGA AAT GTC CAC AAG GTC ACC ACC TGC T GAGCGCCATG         9758
Thr Cys Leu Arg Asn Val His Lys Val Thr Thr Cys
        3135                3140

GTGGGAGAGA CTGTGAGGCG GCAGCTGGGG CCGGAGCCTT TGGAAGTCTG TGCCCTTGTG    9818

CCCTGCCTCC ACCGAGCCAG CTTGGTCCCT ATGGGCTTCC GCACATGCCG CGGGCGGCCA    9878

GGCAACGTGC GTGTCTCTGC CATGTGGCAG AAGTGCTCTT TGTGGCAGTG GCCAGGCAGG    9938

GAGTGTCTGC AGTCCTGGTG GGGCTGAGCC TGAGGCCTTC CAGAAAGCAG GAGCAGCTGT    9998

GCTGCACCCC ATGTGGGTGA CCAGGTCCTT TCTCCTGATA GTCACCTGCT GGTTGTTGCC   10058

AGGTTGCAGC TGCTCTTGCA TCTGGGCCAG AAGTCCTCCC TCCTGCAGGC TGGCTGTTGG   10118

CCCCTCTGCT GTCCTGCAGT AGAAGGTGCC GTGAGCAGGC TTTGGGAACA CTGGCCTGGG   10178

TCTCCCTGGT GGGGTGTGCA TGCCACGCCC CGTGTCTGGA TGCACAGATG CCATGGCCTG   10238

TGCTGGGCCA GTGGCTGGGG GTGCTAGACA CCCGGCACCA TTCTCCCTTC TCTCTTTTCT   10298

TCTCAGGATT TAAAATTTAA TTATATCAGT AAAGAGATTA ATTTTAACGT AAAAAAAAAA   10358

AAAAAAAA                                                            10366
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(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 3144 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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Met Ala Thr Leu Glu Lys Leu Met Lys Ala Phe Glu Ser Leu Lys Ser
 1               5                  10                  15

Phe Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln
            20                  25                  30

Gln Gln Gln Gln Gln Gln Gln Gln Pro Pro Pro Pro Pro Pro Pro Pro
        35                  40                  45

Pro Pro Pro Gln Leu Pro Gln Pro Pro Pro Gln Ala Gln Pro Leu Leu
    50                  55                  60

Pro Gln Pro Gln Pro Pro Pro Pro Pro Pro Pro Pro Pro Gly Pro
65                  70                  75                  80

Ala Val Ala Glu Glu Pro Leu His Arg Pro Lys Lys Glu Leu Ser Ala
                85                  90                  95

Thr Lys Lys Asp Arg Val Asn His Cys Leu Thr Ile Cys Glu Asn Ile
            100                 105                 110

Val Ala Gln Ser Val Arg Asn Ser Pro Glu Phe Gln Lys Leu Leu Gly
        115                 120                 125

Ile Ala Met Glu Leu Phe Leu Leu Cys Ser Asp Asp Ala Glu Ser Asp
    130                 135                 140

Val Arg Met Val Ala Asp Glu Cys Leu Asn Lys Val Ile Lys Ala Leu
145                 150                 155                 160

Met Asp Ser Asn Leu Pro Arg Leu Gln Leu Glu Leu Tyr Lys Glu Ile
                165                 170                 175

Lys Lys Asn Gly Ala Pro Arg Ser Leu Arg Ala Ala Leu Trp Arg Phe
            180                 185                 190

Ala Glu Leu Ala His Leu Val Arg Pro Gln Lys Cys Arg Pro Tyr Leu
        195                 200                 205

Val Asn Leu Leu Pro Cys Leu Thr Arg Thr Ser Lys Arg Pro Glu Glu
    210                 215                 220

Ser Val Gln Glu Thr Leu Ala Ala Ala Val Pro Lys Ile Met Ala Ser
225                 230                 235                 240

Phe Gly Asn Phe Ala Asn Asp Asn Glu Ile Lys Val Leu Leu Lys Ala
                245                 250                 255

Phe Ile Ala Asn Leu Lys Ser Ser Ser Pro Thr Ile Arg Arg Thr Ala
            260                 265                 270

Ala Gly Ser Ala Val Ser Ile Cys Gln His Ser Arg Arg Thr Gln Tyr
        275                 280                 285

Phe Tyr Ser Trp Leu Leu Asn Val Leu Leu Gly Leu Leu Val Pro Val
    290                 295                 300

Glu Asp Glu His Ser Thr Leu Leu Ile Leu Gly Val Leu Leu Thr Leu
305                 310                 315                 320

Arg Tyr Leu Val Pro Leu Leu Gln Gln Gln Val Lys Asp Thr Ser Leu
                325                 330                 335

Lys Gly Ser Phe Gly Val Thr Arg Lys Glu Met Glu Val Ser Pro Ser
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340 345 350

Ala Glu Gln Leu Val Gln Val Tyr Glu Leu Thr Leu His His Thr Gln
355 360 365

His Gln Asp His Asn Val Val Thr Gly Ala Leu Glu Leu Leu Gln Gln
370 375 380

Leu Phe Arg Thr Pro Pro Pro Glu Leu Leu Gln Thr Leu Thr Ala Val
385 390 395 400

Gly Gly Ile Gly Gln Leu Thr Ala Ala Lys Glu Glu Ser Gly Gly Arg
405 410 415

Ser Arg Ser Gly Ser Ile Val Glu Leu Ile Ala Gly Gly Gly Ser Ser
420 425 430

Cys Ser Pro Val Leu Ser Arg Lys Gln Lys Gly Lys Val Leu Leu Gly
435 440 445

Glu Glu Glu Ala Leu Glu Asp Asp Ser Glu Ser Arg Ser Asp Val Ser
450 455 460

Ser Ser Ala Leu Thr Ala Ser Val Lys Asp Glu Ile Ser Gly Glu Leu
465 470 475 480

Ala Ala Ser Ser Gly Val Ser Thr Pro Gly Ser Ala Gly His Asp Ile
485 490 495

Ile Thr Glu Gln Pro Arg Ser Gln His Thr Leu Gln Ala Asp Ser Leu
500 505 510

Asp Leu Ala Ser Cys Asp Leu Thr Ser Ser Ala Thr Asp Gly Asp Glu
515 520 525

Glu Asp Ile Leu Ser His Ser Ser Ser Gln Val Ser Ala Val Pro Ser
530 535 540

Asp Pro Ala Met Asp Leu Asn Asp Gly Thr Gln Ala Ser Ser Pro Ile
545 550 555 560

Ser Asp Ser Ser Gln Thr Thr Thr Glu Gly Pro Asp Ser Ala Val Thr
565 570 575

Pro Ser Asp Ser Ser Glu Ile Val Leu Asp Gly Thr Asp Asn Gln Tyr
580 585 590

Leu Gly Leu Gln Ile Gly Gln Pro Gln Asp Glu Asp Glu Glu Ala Thr
595 600 605

Gly Ile Leu Pro Asp Glu Ala Ser Glu Ala Phe Arg Asn Ser Ser Met
610 615 620

Ala Leu Gln Gln Ala His Leu Leu Lys Asn Met Ser His Cys Arg Gln
625 630 635 640

Pro Ser Asp Ser Ser Val Asp Lys Phe Val Leu Arg Asp Glu Ala Thr
645 650 655

Glu Pro Gly Asp Gln Glu Asn Lys Pro Cys Arg Ile Lys Gly Asp Ile
660 665 670

Gly Gln Ser Thr Asp Asp Asp Ser Ala Pro Leu Val His Ser Val Arg
675 680 685

Leu Leu Ser Ala Ser Phe Leu Leu Thr Gly Gly Lys Asn Val Leu Val
690 695 700

Pro Asp Arg Asp Val Arg Val Ser Val Lys Ala Leu Ala Leu Ser Cys
705 710 715 720

Val Gly Ala Ala Val Ala Leu His Pro Glu Ser Phe Phe Ser Lys Leu
725 730 735

Tyr Lys Val Pro Leu Asp Thr Thr Glu Tyr Pro Glu Glu Gln Tyr Val
740 745 750

Ser Asp Ile Leu Asn Tyr Ile Asp His Gly Asp Pro Gln Val Arg Gly
755 760 765

Ala Thr Ala Ile Leu Cys Gly Thr Leu Ile Cys Ser Ile Leu Ser Arg
770 775 780

Ser Arg Phe His Val Gly Asp Trp Met Gly Thr Ile Arg Thr Leu Thr
785 790 795 800

Gly Asn Thr Phe Ser Leu Ala Asp Cys Ile Pro Leu Leu Arg Lys Thr
805 810 815

Leu Lys Asp Glu Ser Ser Val Thr Cys Lys Leu Ala Cys Thr Ala Val
820 825 830

Arg Asn Cys Val Met Ser Leu Cys Ser Ser Ser Tyr Ser Glu Leu Gly
835 840 845

Leu Gln Leu Ile Ile Asp Val Leu Thr Leu Arg Asn Ser Ser Tyr Trp
850 855 860

Leu Val Arg Thr Glu Leu Leu Glu Thr Leu Ala Glu Ile Asp Phe Arg
865 870 875 880

Leu Val Ser Phe Leu Glu Ala Lys Ala Glu Asn Leu His Arg Gly Ala
885 890 895

His His Tyr Thr Gly Leu Leu Lys Leu Gln Glu Arg Val Leu Asn Asn
900 905 910

Val Val Ile His Leu Leu Gly Asp Glu Asp Pro Arg Val Arg His Val
915 920 925

Ala Ala Ala Ser Leu Ile Arg Leu Val Pro Lys Leu Phe Tyr Lys Cys
930 935 940

Asp Gln Gly Gln Ala Asp Pro Val Val Ala Val Ala Arg Asp Gln Ser
945 950 955 960

Ser Val Tyr Leu Lys Leu Leu Met His Glu Thr Gln Pro Pro Ser His
965 970 975

Phe Ser Val Ser Thr Ile Thr Arg Ile Tyr Arg Gly Tyr Asn Leu Leu
980 985 990

Pro Ser Ile Thr Asp Val Thr Met Glu Asn Asn Leu Ser Arg Val Ile
995 1000 1005

Ala Ala Val Ser His Glu Leu Ile Thr Ser Thr Thr Arg Ala Leu Thr
1010 1015 1020

Phe Gly Cys Cys Glu Ala Leu Cys Leu Leu Ser Thr Ala Phe Pro Val
1025 1030 1035 1040

Cys Ile Trp Ser Leu Gly Trp His Cys Gly Val Pro Pro Leu Ser Ala
1045 1050 1055

Ser Asp Glu Ser Arg Lys Ser Cys Thr Val Gly Met Ala Thr Met Ile
1060 1065 1070

Leu Thr Leu Leu Ser Ser Ala Trp Phe Pro Leu Asp Leu Ser Ala His
1075 1080 1085

Gln Asp Ala Leu Ile Leu Ala Gly Asn Leu Leu Ala Ala Ser Ala Pro
1090 1095 1100

Lys Ser Leu Arg Ser Ser Trp Ala Ser Glu Glu Glu Ala Asn Pro Ala
1105 1110 1115 1120

Ala Thr Lys Gln Glu Glu Val Trp Pro Ala Leu Gly Asp Arg Ala Leu
1125 1130 1135

Val Pro Met Val Glu Gln Leu Phe Ser His Leu Leu Lys Val Ile Asn
1140 1145 1150

Ile Cys Ala His Val Leu Asp Asp Val Ala Pro Gly Pro Ala Ile Lys
1155 1160 1165

Ala Ala Leu Pro Ser Leu Thr Asn Pro Pro Ser Leu Ser Pro Ile Arg
1170 1175 1180

Arg Lys Gly Lys Glu Lys Glu Pro Gly Glu Gln Ala Ser Val Pro Leu
1185 1190 1195 1200

Ser Pro Lys Lys Gly Ser Glu Ala Ser Ala Ala Ser Arg Gln Ser Asp
1205 1210 1215

Thr Ser Gly Pro Val Thr Thr Ser Lys Ser Ser Ser Leu Gly Ser Phe
1220 1225 1230

Tyr His Leu Pro Ser Tyr Leu Arg Leu His Asp Val Leu Lys Ala Thr
1235 1240 1245

His Ala Asn Tyr Lys Val Thr Leu Asp Leu Gln Asn Ser Thr Glu Lys
1250 1255 1260

Phe Gly Gly Phe Leu Arg Ser Ala Leu Asp Val Leu Ser Gln Ile Leu
1265 1270 1275 1280

Glu Leu Ala Thr Leu Gln Asp Ile Gly Lys Cys Val Glu Glu Ile Leu
1285 1290 1295

Gly Tyr Leu Lys Ser Cys Phe Ser Arg Glu Pro Met Met Ala Thr Val
1300 1305 1310

Cys Val Gln Gln Leu Leu Lys Thr Leu Phe Gly Thr Asn Leu Ala Ser
1315 1320 1325

Gln Phe Asp Gly Leu Ser Ser Asn Pro Ser Lys Ser Gln Gly Arg Ala
1330 1335 1340

Gln Arg Leu Gly Ser Ser Ser Val Arg Pro Gly Leu Tyr His Tyr Cys
1345 1350 1355 1360

Phe Met Ala Pro Tyr Thr His Phe Thr Gln Ala Leu Ala Asp Ala Ser
1365 1370 1375

Leu Arg Asn Met Val Gln Ala Glu Gln Glu Asn Asp Thr Ser Gly Trp
1380 1385 1390

Phe Asp Val Leu Gln Lys Val Ser Thr Gln Leu Lys Thr Asn Leu Thr
1395 1400 1405

Ser Val Thr Lys Asn Arg Ala Asp Lys Asn Ala Ile His Asn His Ile
1410 1415 1420

Arg Leu Phe Glu Pro Leu Val Ile Lys Ala Leu Lys Gln Tyr Thr Thr
1425 1430 1435 1440

Thr Thr Cys Val Gln Leu Gln Lys Gln Val Leu Asp Leu Leu Ala Gln
1445 1450 1455

Leu Val Gln Leu Arg Val Asn Tyr Cys Leu Leu Asp Ser Asp Gln Val
1460 1465 1470

Phe Ile Gly Phe Val Leu Lys Gln Phe Glu Tyr Ile Glu Val Gly Gln
1475 1480 1485

Phe Arg Glu Ser Glu Ala Ile Ile Pro Asn Ile Phe Phe Phe Leu Val
1490 1495 1500

Leu Leu Ser Tyr Glu Arg Tyr His Ser Lys Gln Ile Ile Gly Ile Pro
1505 1510 1515 1520

Lys Ile Ile Gln Leu Cys Asp Gly Ile Met Ala Ser Gly Arg Lys Ala
1525 1530 1535

Val Thr His Ala Ile Pro Ala Leu Gln Pro Ile Val His Asp Leu Phe
1540 1545 1550

Val Leu Arg Gly Thr Asn Lys Ala Asp Ala Gly Lys Glu Leu Glu Thr
1555 1560 1565

Gln Lys Glu Val Val Val Ser Met Leu Leu Arg Leu Ile Gln Tyr His
1570 1575 1580

Gln Val Leu Glu Met Phe Ile Leu Val Leu Gln Gln Cys His Lys Glu
1585 1590 1595 1600

Asn Glu Asp Lys Trp Lys Arg Leu Ser Arg Gln Ile Ala Asp Ile Ile
1605 1610 1615

Leu Pro Met Leu Ala Lys Gln Gln Met His Ile Asp Ser His Glu Ala
1620 1625 1630

Leu Gly Val Leu Asn Thr Leu Phe Glu Ile Leu Ala Pro Ser Ser Leu
1635 1640 1645

Arg Pro Val Asp Met Leu Leu Arg Ser Met Phe Val Thr Pro Asn Thr
1650 1655 1660

Met Ala Ser Val Ser Thr Val Gln Leu Trp Ile Ser Gly Ile Leu Ala
1665 1670 1675 1680

Ile Leu Arg Val Leu Ile Ser Gln Ser Thr Glu Asp Ile Val Leu Ser
1685 1690 1695

Arg Ile Gln Glu Leu Ser Phe Ser Pro Tyr Leu Ile Ser Cys Thr Val
1700 1705 1710

Ile Asn Arg Leu Arg Asp Gly Asp Ser Thr Ser Thr Leu Glu Glu His
1715 1720 1725

Ser Glu Gly Lys Gln Ile Lys Asn Leu Pro Glu Glu Thr Phe Ser Arg
1730 1735 1740

Phe Leu Leu Gln Leu Val Gly Ile Leu Leu Glu Asp Ile Val Thr Lys
1745 1750 1755 1760

Gln Leu Lys Val Glu Met Ser Glu Gln Gln His Thr Phe Tyr Cys Gln
1765 1770 1775

Glu Leu Gly Thr Leu Leu Met Cys Leu Ile His Ile Phe Lys Ser Gly
1780 1785 1790

Met Phe Arg Arg Ile Thr Ala Ala Ala Thr Arg Leu Phe Arg Ser Asp
1795 1800 1805

Gly Cys Gly Gly Ser Phe Tyr Thr Leu Asp Ser Leu Asn Leu Arg Ala
1810 1815 1820

Arg Ser Met Ile Thr Thr His Pro Ala Leu Val Leu Leu Trp Cys Gln
1825 1830 1835 1840

Ile Leu Leu Leu Val Asn His Thr Asp Tyr Arg Trp Trp Ala Glu Val
1845 1850 1855

Gln Gln Thr Pro Lys Arg His Ser Leu Ser Ser Thr Lys Leu Leu Ser
1860 1865 1870

Pro Gln Met Ser Gly Glu Glu Glu Asp Ser Asp Leu Ala Ala Lys Leu
1875 1880 1885

Gly Met Cys Asn Arg Glu Ile Val Arg Arg Gly Ala Leu Ile Leu Phe
1890 1895 1900

Cys Asp Tyr Val Cys Gln Asn Leu His Asp Ser Glu His Leu Thr Trp
1905 1910 1915 1920

Leu Ile Val Asn His Ile Gln Asp Leu Ile Ser Leu Ser His Glu Pro
1925 1930 1935

Pro Val Gln Asp Phe Ile Ser Ala Val His Arg Asn Ser Ala Ala Ser
1940 1945 1950

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1955 1960 1965

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1970 1975 1980

Ser Gln Ser Gly Ala Val Leu Thr Leu Tyr Val Asp Arg Leu Leu Cys
1985 1990 1995 2000

Thr Pro Phe Arg Val Leu Ala Arg Met Val Asp Ile Leu Ala Cys Arg
2005 2010 2015

Arg Val Glu Met Leu Leu Ala Ala Asn Leu Gln Ser Ser Met Ala Gln
2020 2025 2030

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Ser His Pro Leu Asp Gly Asp Gly His Val Ser Leu Glu Thr Val Ser
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Pro Asp Lys Asp Trp Tyr Val His Leu Val Lys Ser Gln Cys Trp Thr
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2145 2150 2155 2160

Gly Gln Lys Ser Ala Leu Phe Glu Ala Ala Arg Glu Val Thr Leu Ala
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Arg Val Ser Gly Thr Val Gln Gln Leu Pro Ala Val His His Val Phe
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Leu Ser Leu Asp Leu Gln Ala Gly Leu Asp Cys Cys Cys Leu Ala Leu
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Gln Leu Pro Gly Leu Trp Ser Val Val Ser Ser Thr Glu Phe Val Thr
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His Ala Cys Ser Leu Ile Tyr Cys Val His Phe Ile Leu Glu Ala Val
2305 2310 2315 2320

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Thr Pro Lys Ala Ile Ser Glu Glu Glu Glu Glu Val Asp Pro Asn Thr
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Gln Asn Pro Lys Tyr Ile Thr Ala Ala Cys Glu Met Val Ala Glu Met
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Gly Val Pro Ala Phe Leu Thr Pro Leu Leu Arg Asn Ile Ile Ile Ser
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Lys Glu Phe Ile Tyr Arg Ile Asn Thr Leu Gly Trp Thr Ser Arg Thr
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Ala Ile Leu Prc His Val Ile Ser Arg Met Gly Lys Leu Glu Gln Val
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Asp Val Asn Leu Phe Cys Leu Val Ala Thr Asp Phe Tyr Arg His Gln
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Ile Glu Glu Glu Leu Asp Arg Arg Ala Phe Gln Ser Val Leu Glu Val
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Val Ala Ala Pro Gly Ser Pro Tyr His Arg Leu Leu Thr Cys Leu Arg
3125 3130 3135

Asn Val His Lys Val Thr Thr Cys
3140

Claims

1. An isolated, purified or recombinant polypeptide comprising a huntingtin protein or a mutuant, fragment or variant thereof having substantially the same activity as huntingtin protein.

2. A polypeptide according to claim 1 having the amino acid sequence shown in SEQ ID NO:6.

3. A polypeptide according to claim 1 or 2 which is essentially purified and/or has at least 5 contiguous amino acids.

4. An isolated, purified or recombinant nucleic acid molecule comprising nucleic acid which is:
   (a) a sequence encoding a huntingtin protein according to any preceding claim (whether normal or genetically defective), or its complementary strand;
   (b) a sequence that is substantially homologous to, or hybridises under stringent conditions to, either sequence in (a);
   (c) a sequence that is substantially homologous to, or would hybridise under stringent conditions to, a sequence in (a) or (b) but for the degeneracy of the genetic code;
   or a fragment of any of (a), (b) or (c).

5. A nucleic acid according to claim 1, wherein the huntingtin protein has the amino acid sequence shown in SEQ ID NO:6 and/or the nucleic acid is DNA encoding the amino acid sequence SEQ ID NO:5.

6. A nucleic acid molecule according to claim 4 or 5 which is a probe for detecting the presence of huntingtin in a sample comprising being at least 5, such as at least 15, contiguous nucleotides.

7. A (preferably recombinant) nucleic acid molecule according to any of claims 4 to 6 comprising a transcriptional region functional in a cell operably linked to a sequence complimentary to an RNA sequence encoding a protein according to any of claims 1 to 3 or at least 5 contiguous amino acids thereof.

8. A vector comprising a nucleic acid molecule according to any of claims 4 to 7.

9. A vector according to claim 8 wherein the nucleic acid molecule, such as encoding huntingtin protein, is operably linked to transcriptional and/or translational expression signals.

10. A host cell transformed or transfected with a vector according to claim 4 or 5.

11. An antibody specific for huntingtin protein, or a protein according to any of claims 1 to 3.

12. A hybridoma which produces an antibody according to claim 11.

13. A method of detecting the presence of, or predisposition to develop, Huntington's disease in a subject, the method comprising evaluating the characteristics of huntingtin nucleic acid in a sample from the subject, for example in relation to the number of (CAG) repeats.

14. A method according to claim 13 comprising:
   (a) taking a sample from the subject;
   (b) evaluating the characteristics of huntingtin nucleic acid in the sample, wherein the evaluation comprises detecting the huntingtin $(CAG)_n$ region in the sample; and
   (c) comparing the characteristics found in (b) with a similar analysis from an individual not having, or not suspected of having, Huntington's disease; and
   (d) the presence of, or predisposition to develop, Huntington's disease being indicated if those characteristics in the huntingtin $(CAG)_n$ region differ.

15. A method according to claim 13 comprising:
   (a) taking a sample from a subject and;
   (b) evaluating the characteristics of huntingtin nucleic acid comprising the huntingtin $(CAG)_n$ region in the sample by Southern blot, northern blot, or polymerase chain reaction analysis.

16. The use of:
(a) a nucleic acid molecule according to any of claims 4 to 6 or a vector according to claim 8 which encodes a functional (or non-defective) protein;
(b) a polypeptide according to any of claims 1 to 3 which is functional (or non-defective);
(c) a host cell according to claim 10 expressing a polypeptide which is functional (or non-defective); and/or
(d) an antagonist to, or a compound that binds to, huntingdon protein;
in the preparation of an agent for treating, delaying or preventing a neurodegenerative disorder.

17. The use according to claim 16 which is gene therapy.

18. The use according to claim 16 or 17 for treating, preventing or delaying Huntingdon's disease.

19. The use according to any of claims 16 to 17 wherein the nucleic acid has from 11 to 34 (CAG) repeats and/or the polypeptide has from 11 to 34 Gln repeats, said repeats being consecutive.

20. A diagnostic and/or immunoassay kit comprising at least one container and;
(a) a nucleic acid molecule according to any of claims 4 to 6, optionally labelled; or
(b) an antibody according to claim 11, optionally labelled.

21. The use of:
(a) a nucleic acid molecule according to any of claims 4 to 6 or a vector according to claim 8 which encodes a functional (or non-defective) protein;
(b) a polypeptide according to any of claims 1 to 3 which is functional (or non-defective);
(c) a host cell according to claim 10 expressing a polypeptide which is functional (or non-defective); and/or
(d) an antagonist to, or a compound that binds to, huntingdon protein;
in the preparation of a medicament.

22. A pharmaceutical composition comprising:
(a) a nucleic acid molecule according to any of claims 4 to 6 or a vector according to claim 8 which encodes a functional (or non-defective) protein;
(b) a polypeptide according to any of claims 1 to 3 which is functional (or non-defective);
(c) a host cell according to claim 10 expressing a polypeptide which is functional (or non-defective); and/or
(d) an antagonist to, or a compound that binds to, huntingdon protein;
in admixture with pharmaceutically acceptable carrier.

23. A process for the preparation of a polypeptide, the process comprising culturing a host cell according to claim 10 under conditions whereby the polypeptide is expressed, and purifying or isolating the polypeptide.

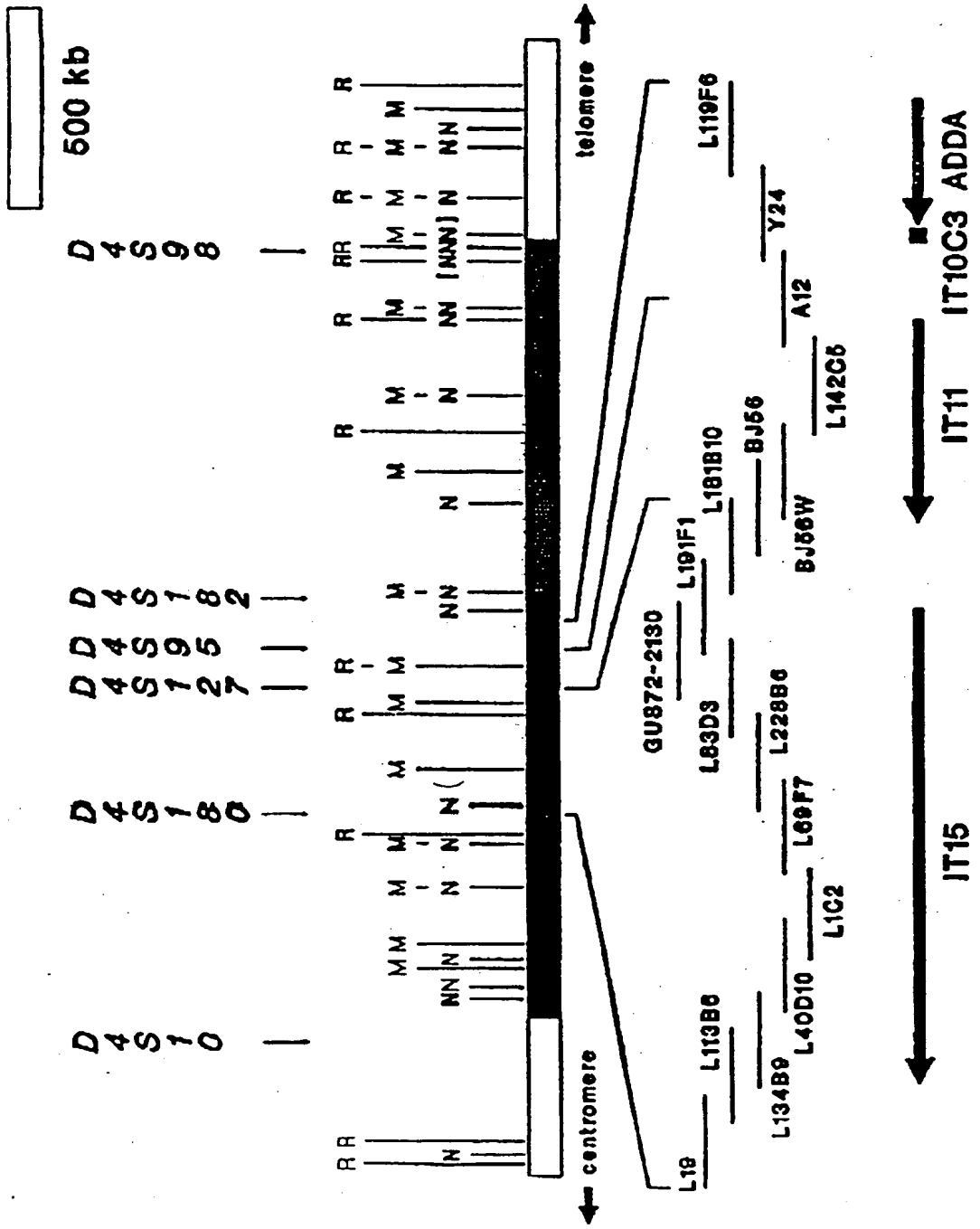


FIGURE 1

Figure 2

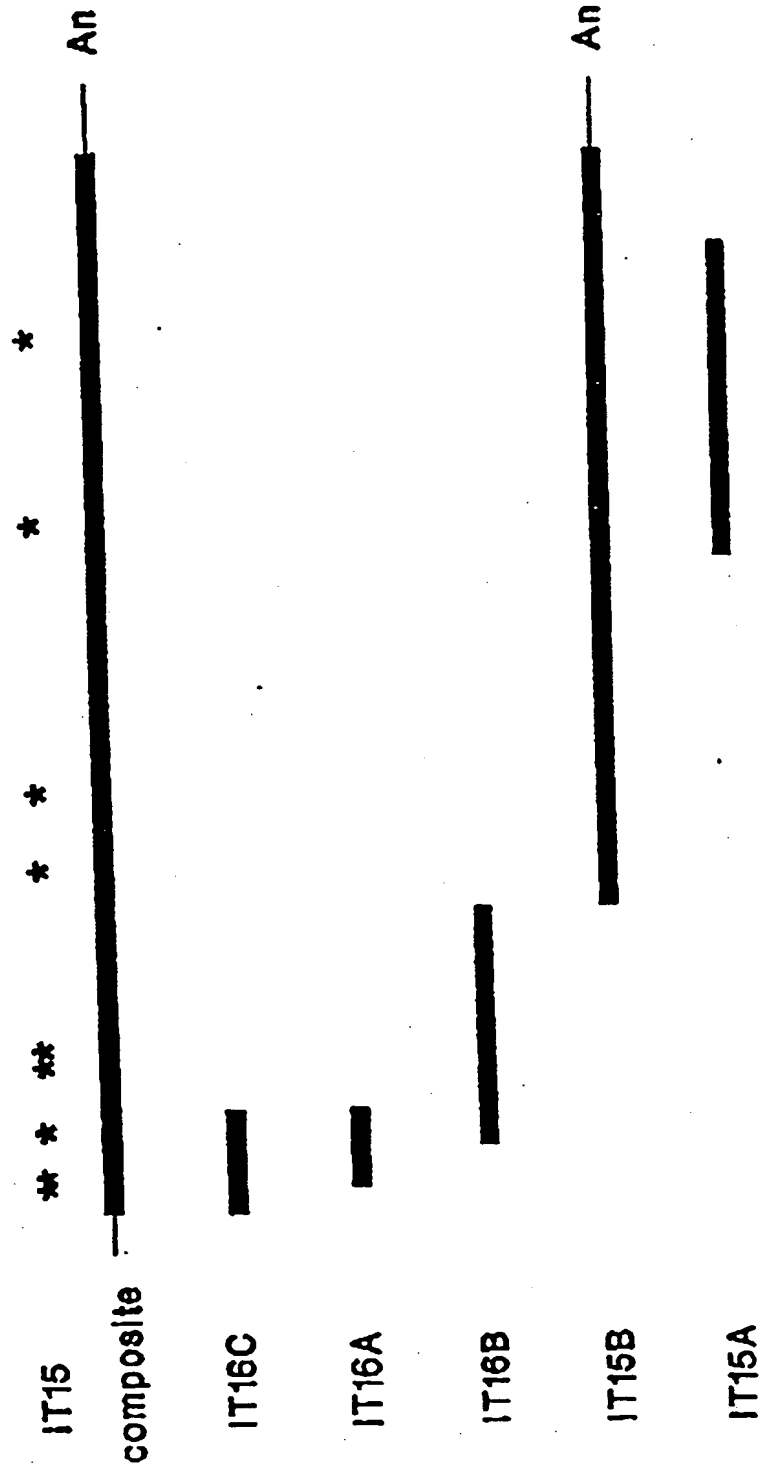


Figura 3

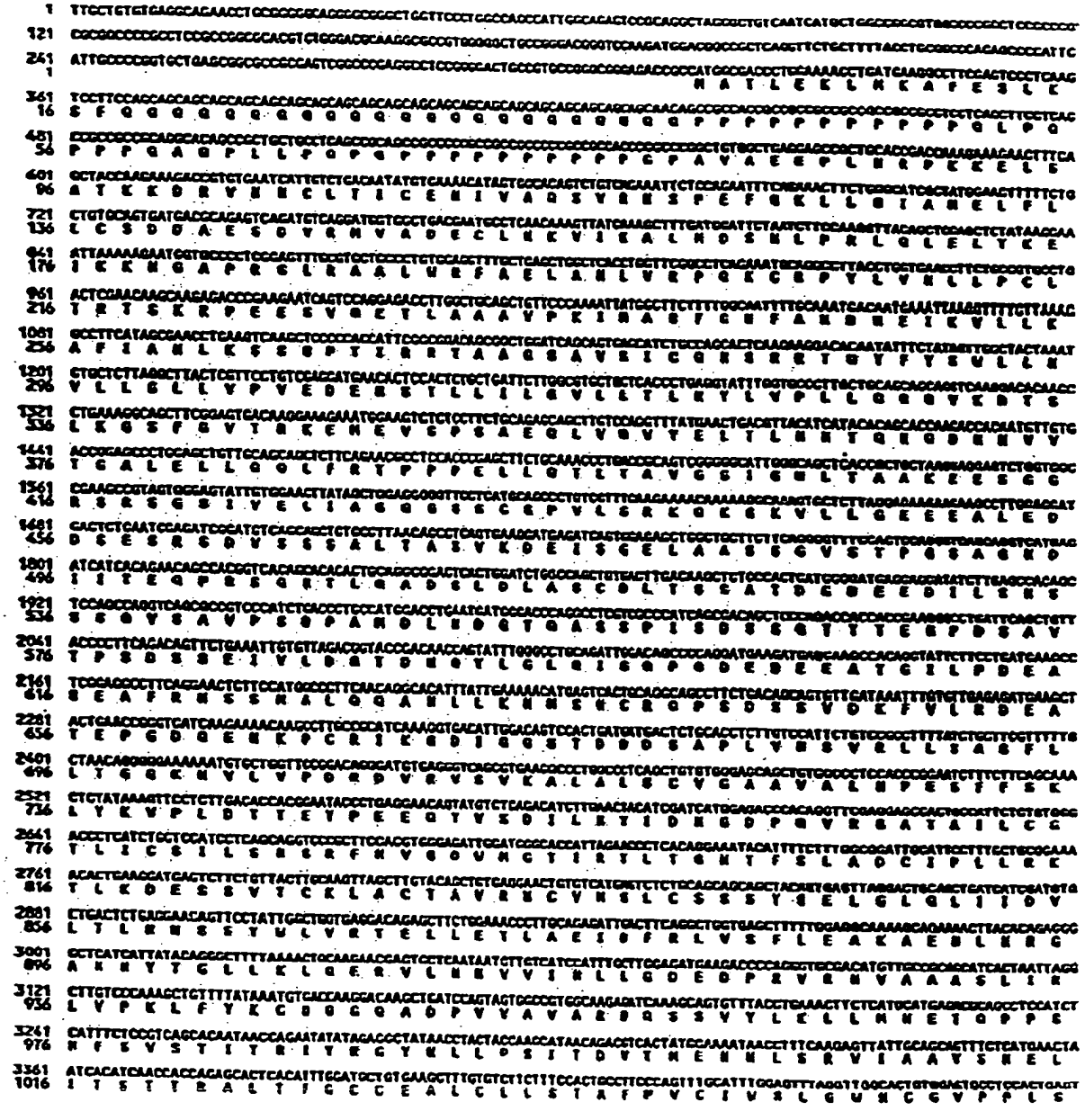


FIGURE 4 (Sheet 1 of 3)

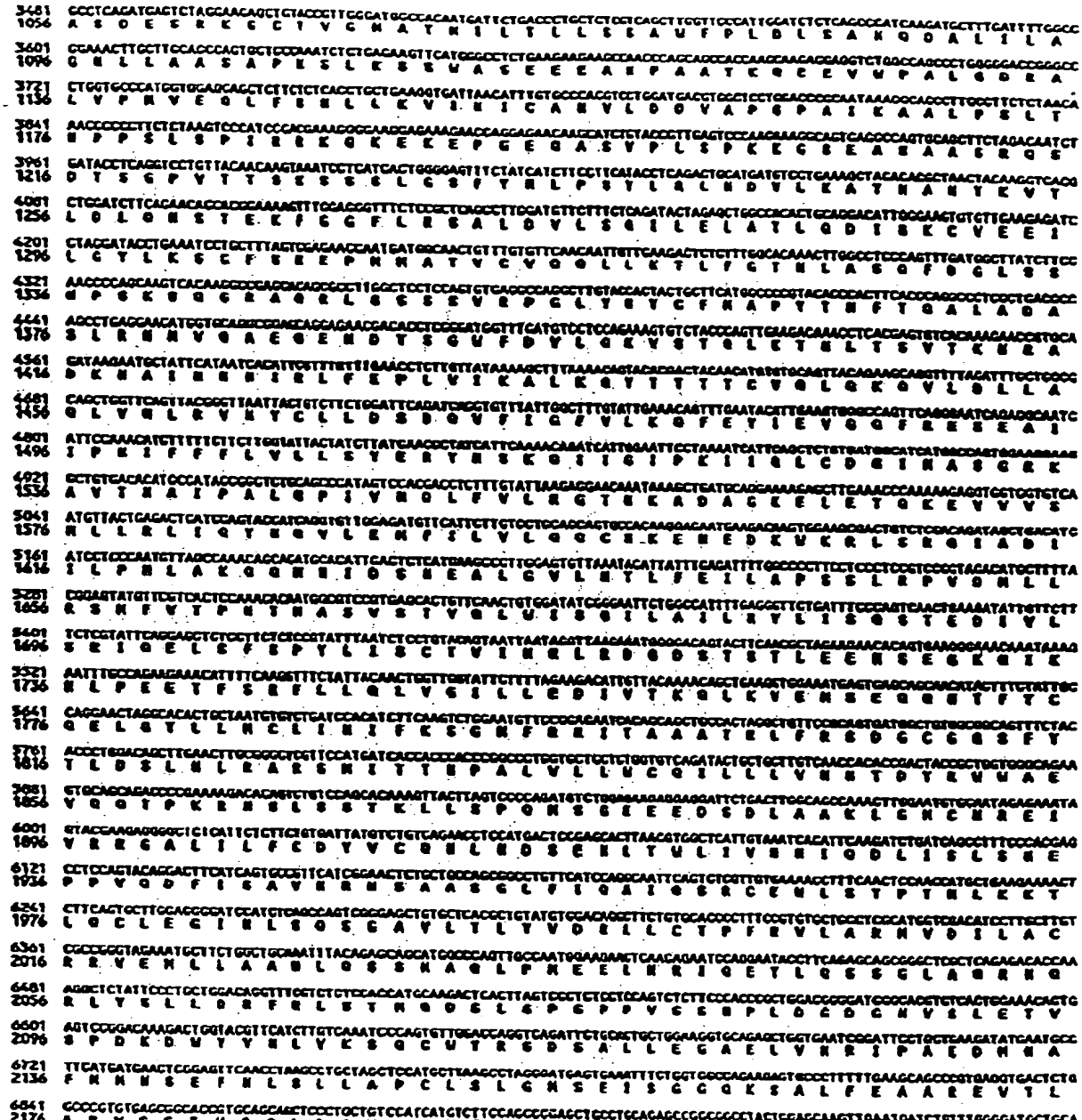


FIGURE 4 (Sheet 2 of 3)

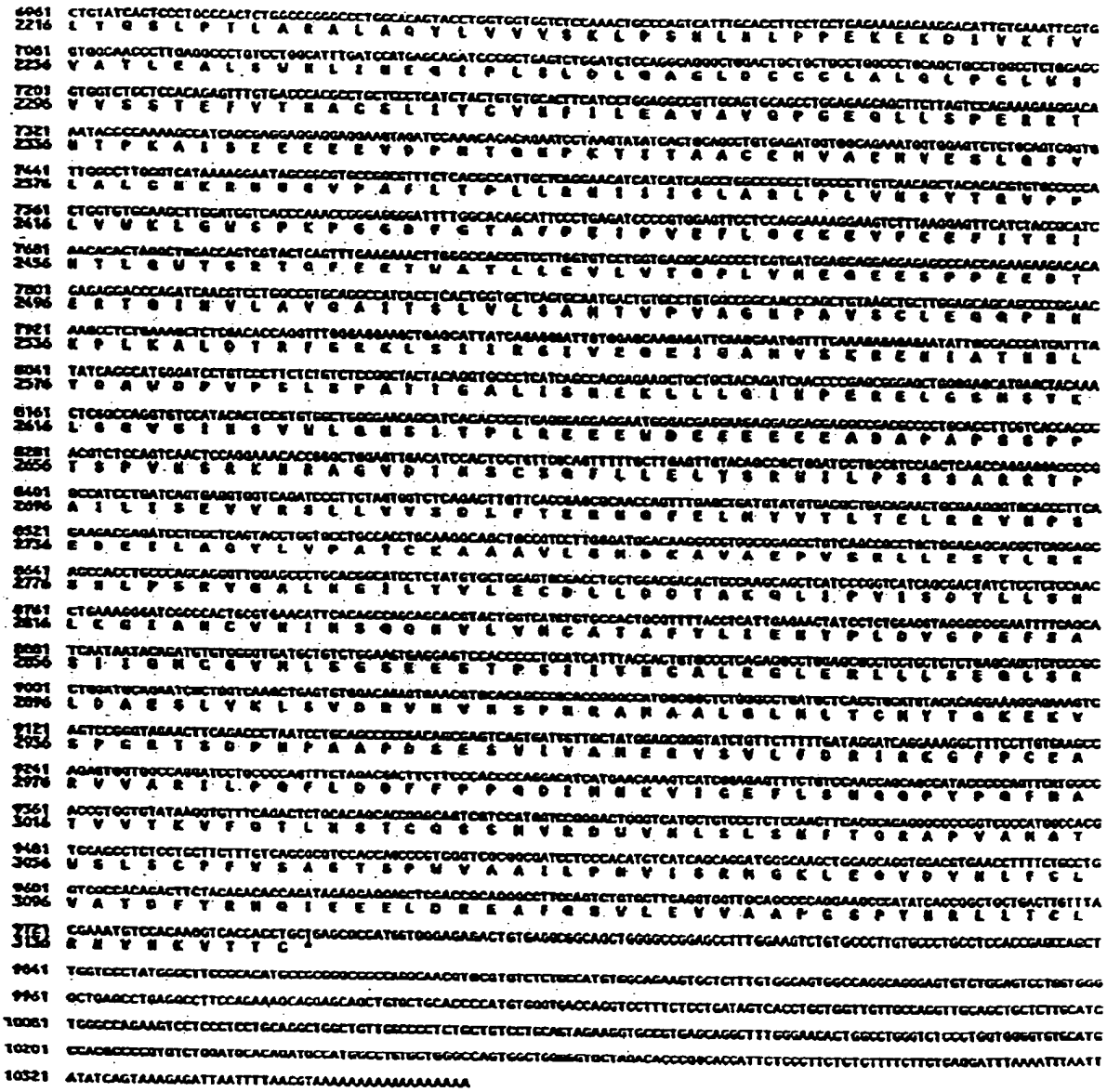


FIGURE 4
(sheet 3 of 3)

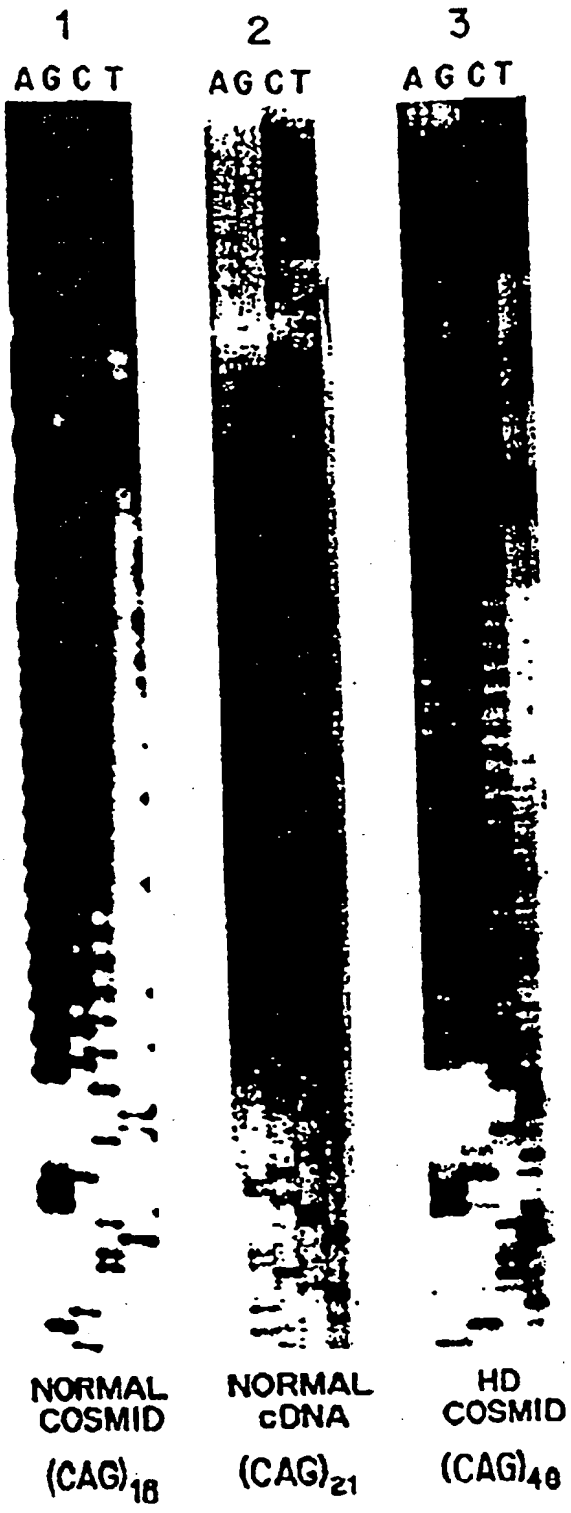


FIGURE 5

FIGURE 6

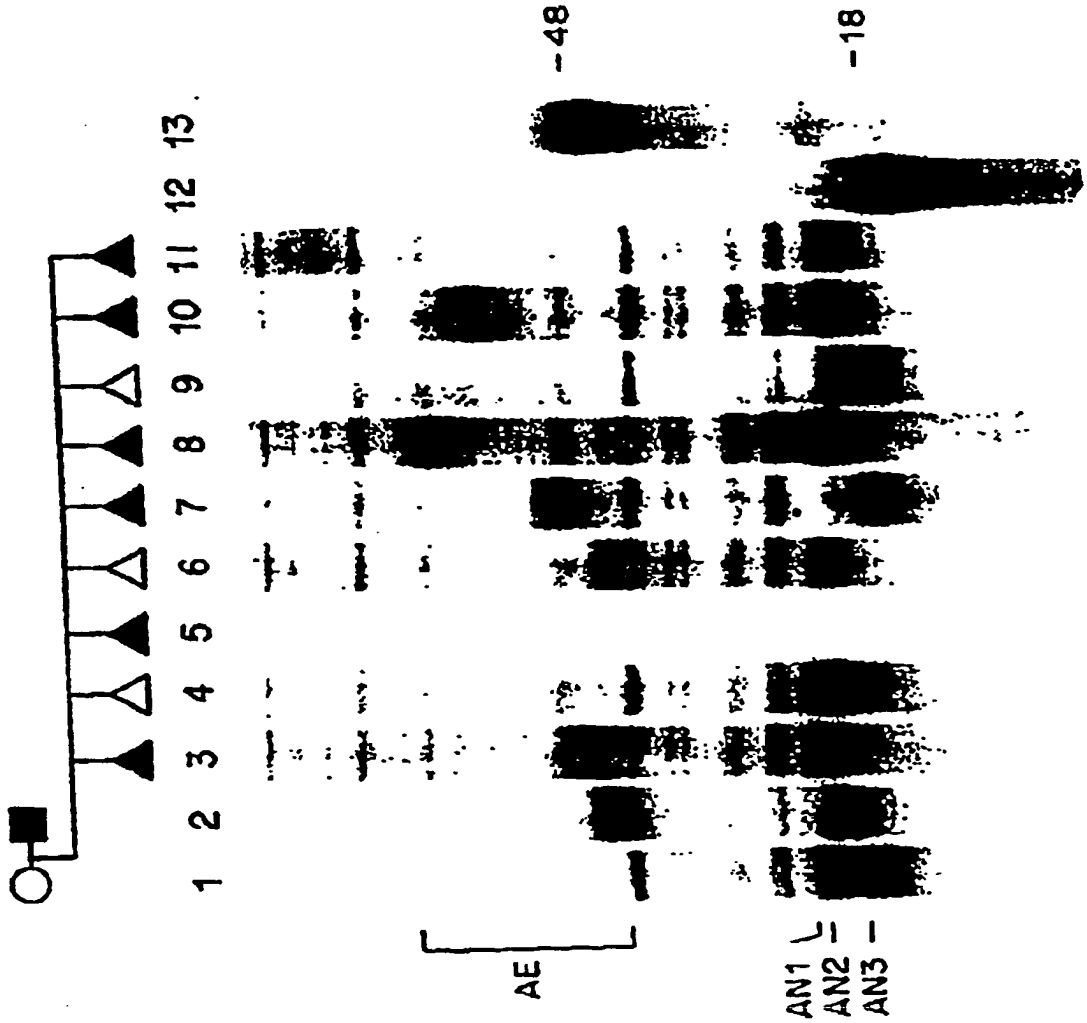


FIGURE 7

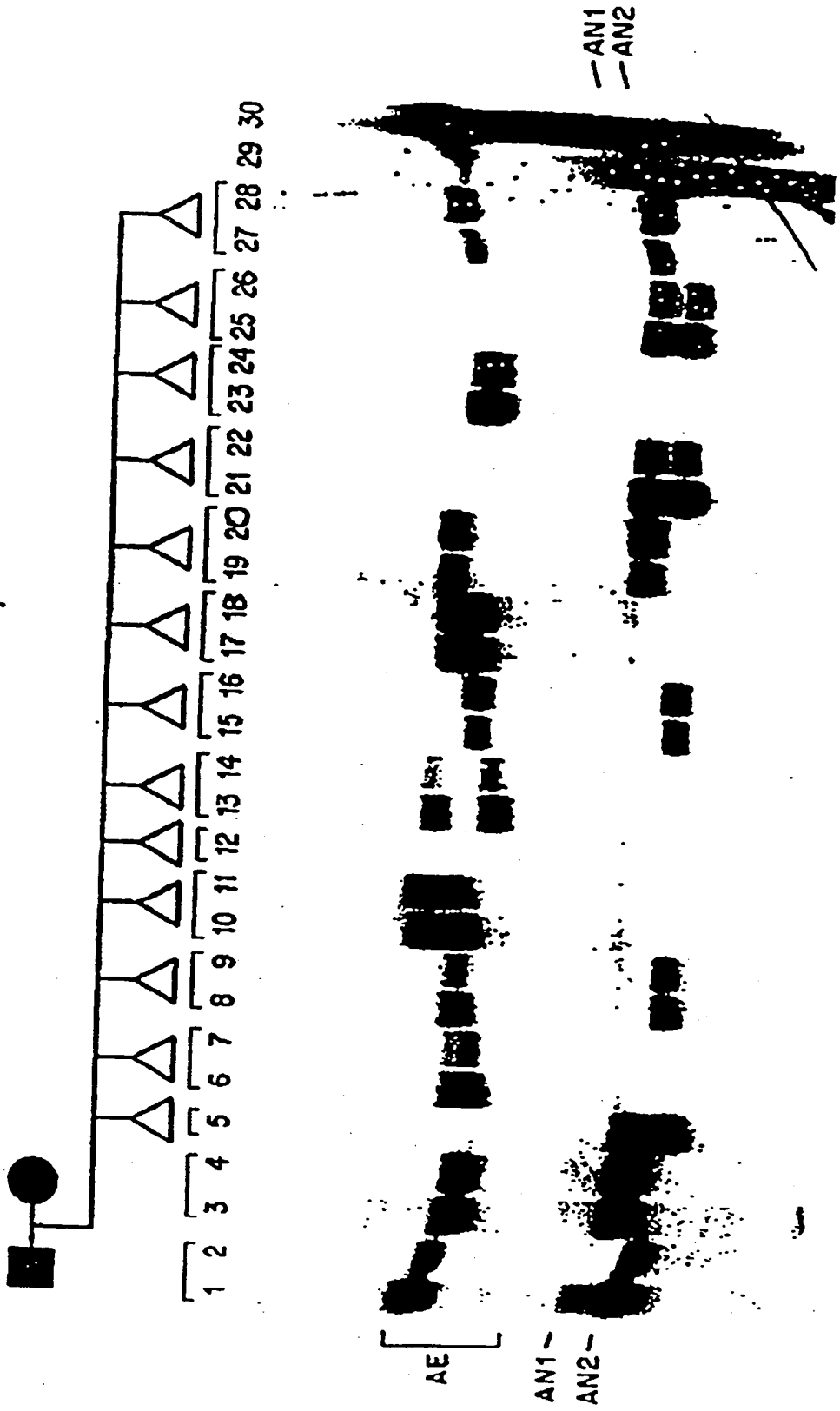


FIGURE 8

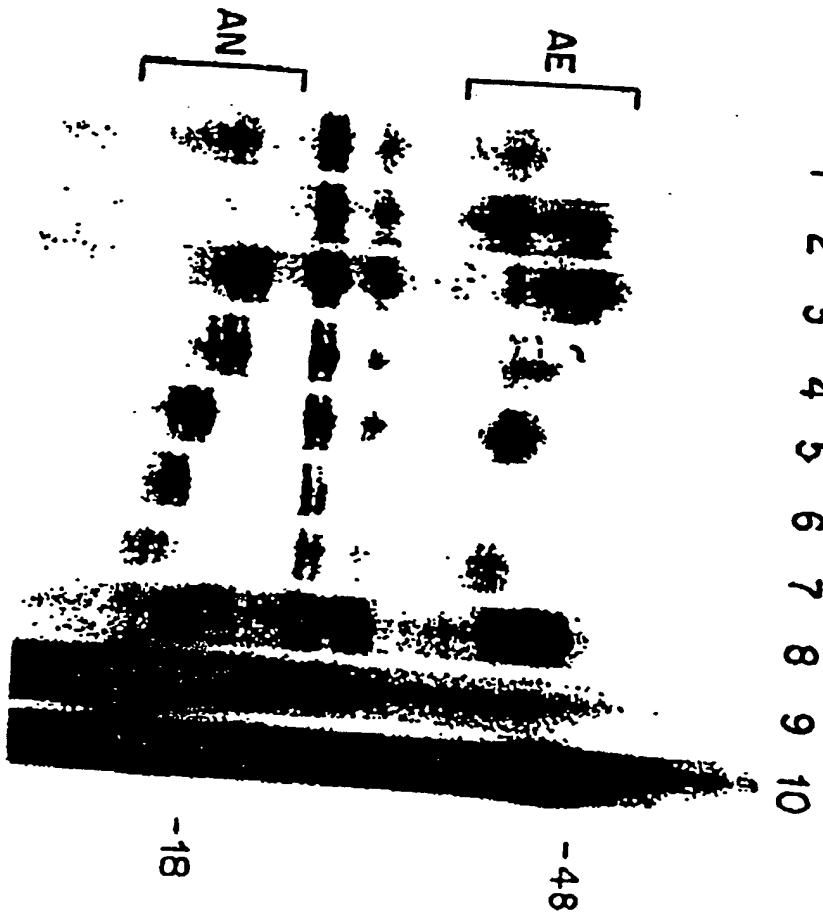


FIGURE 9

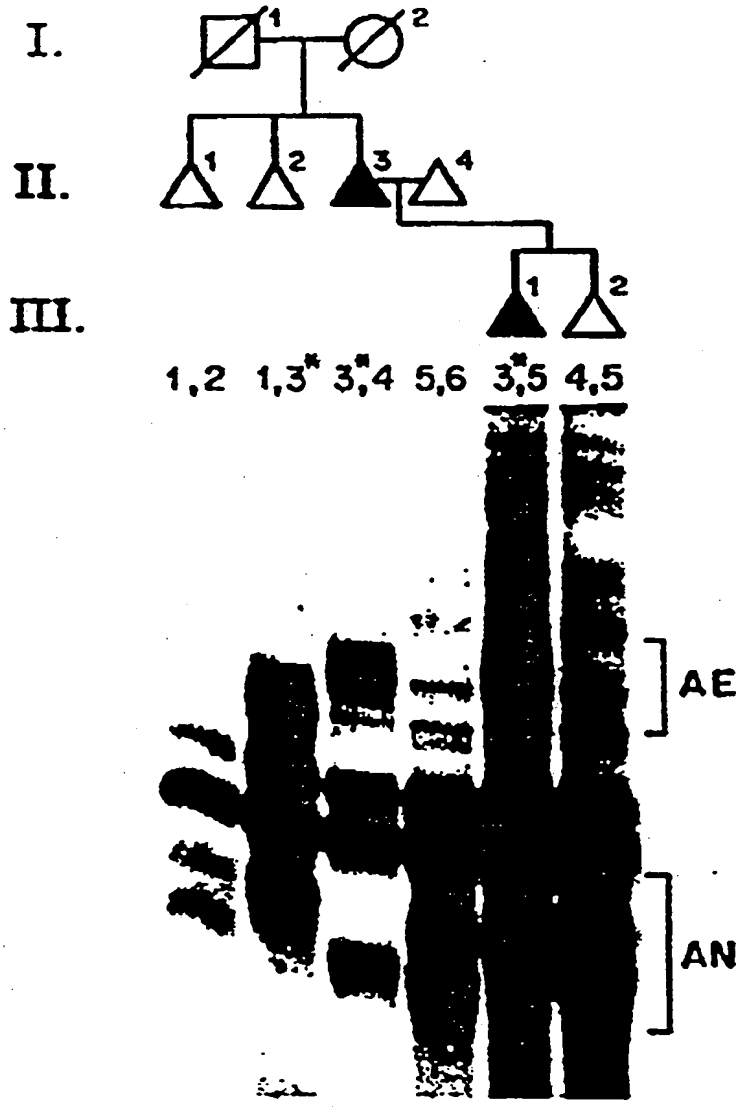


FIGURE 10

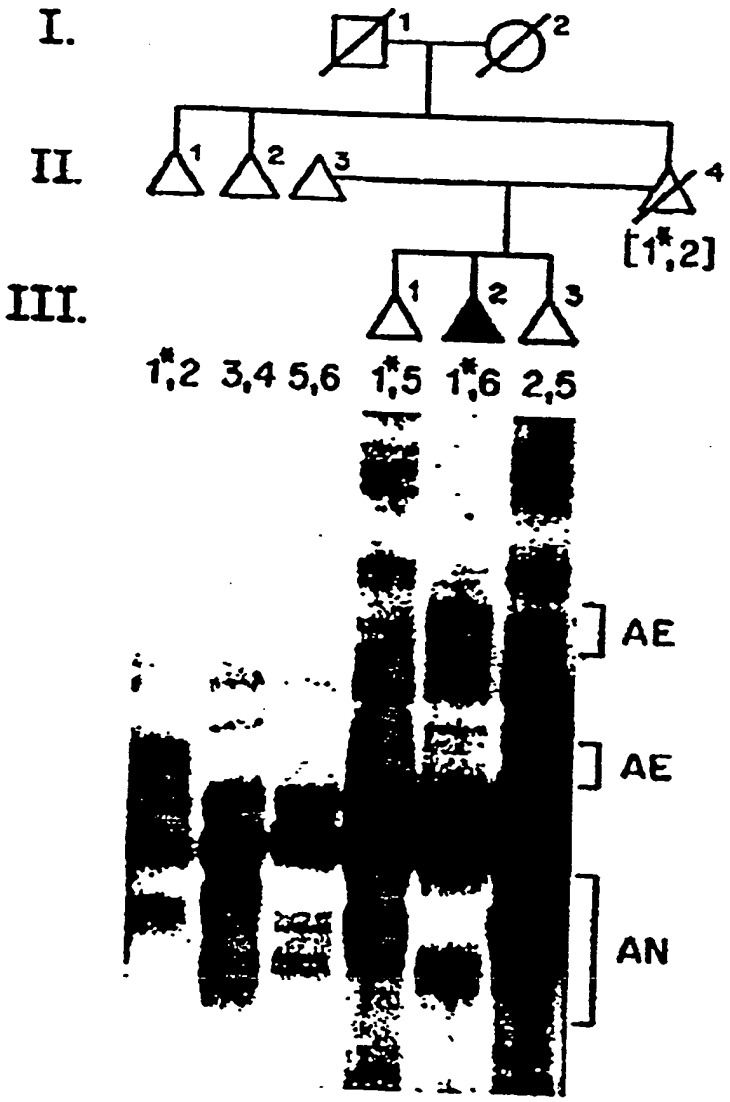
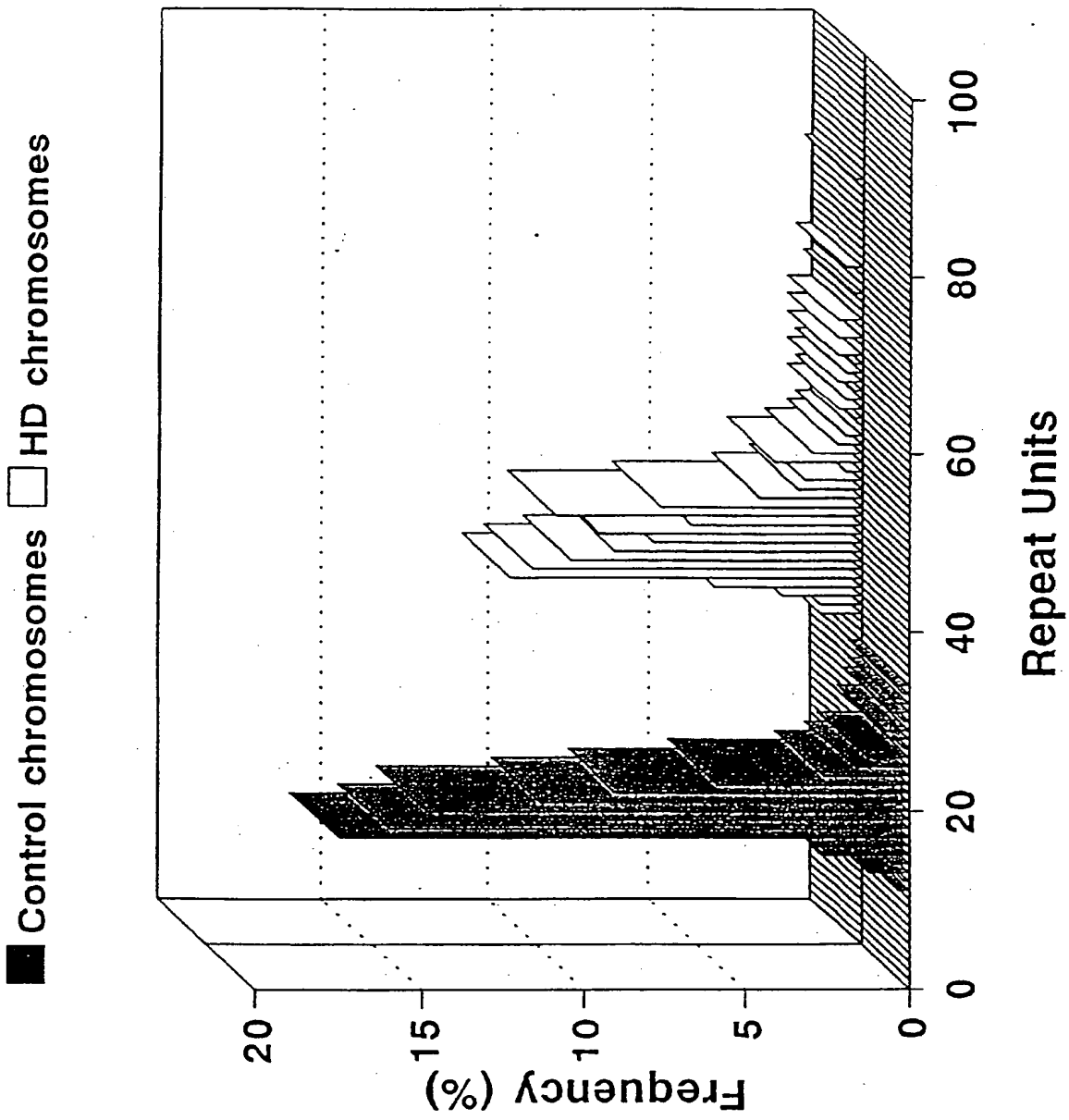


FIGURE 11



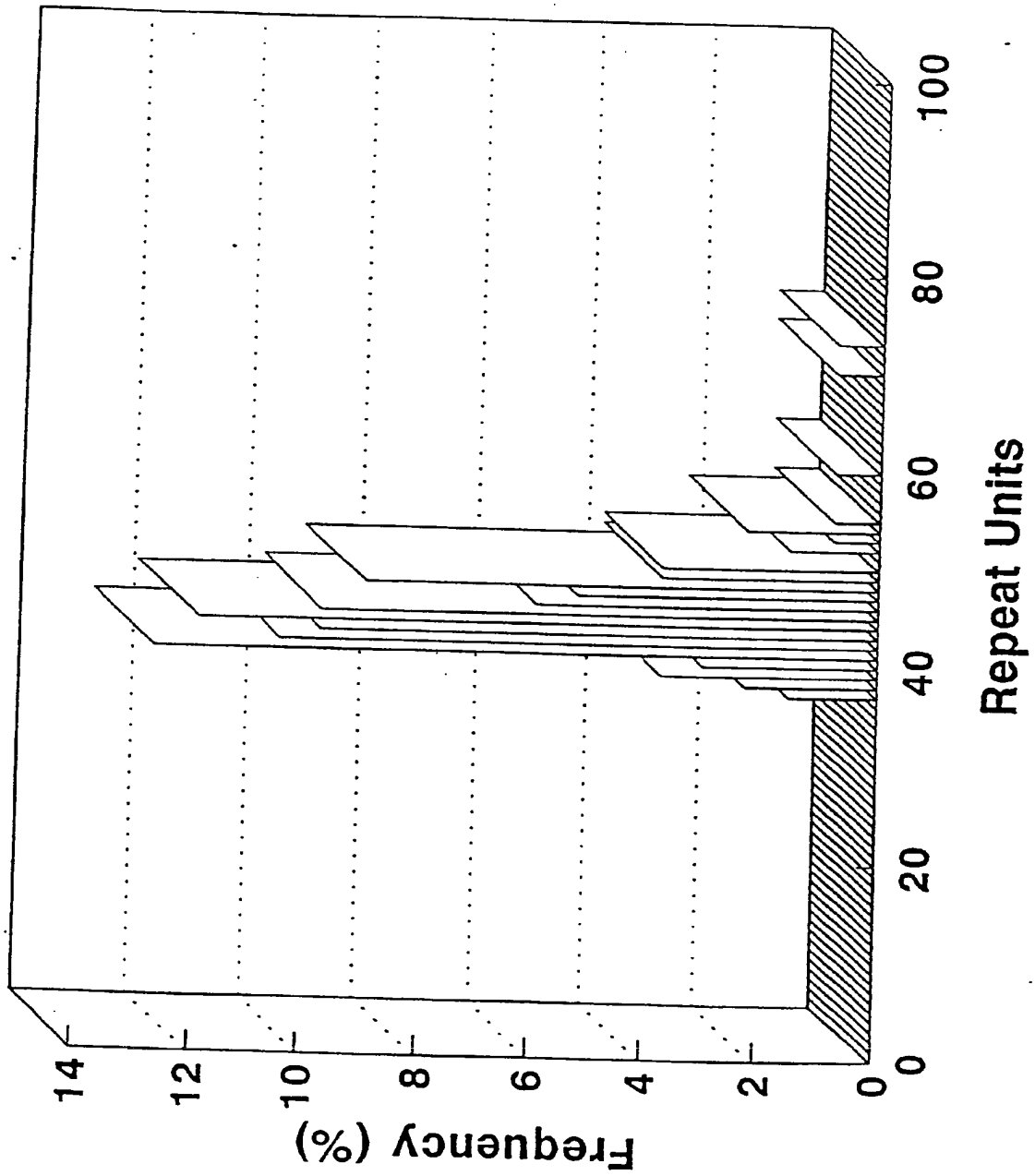


FIGURE 12 (PANEL A)

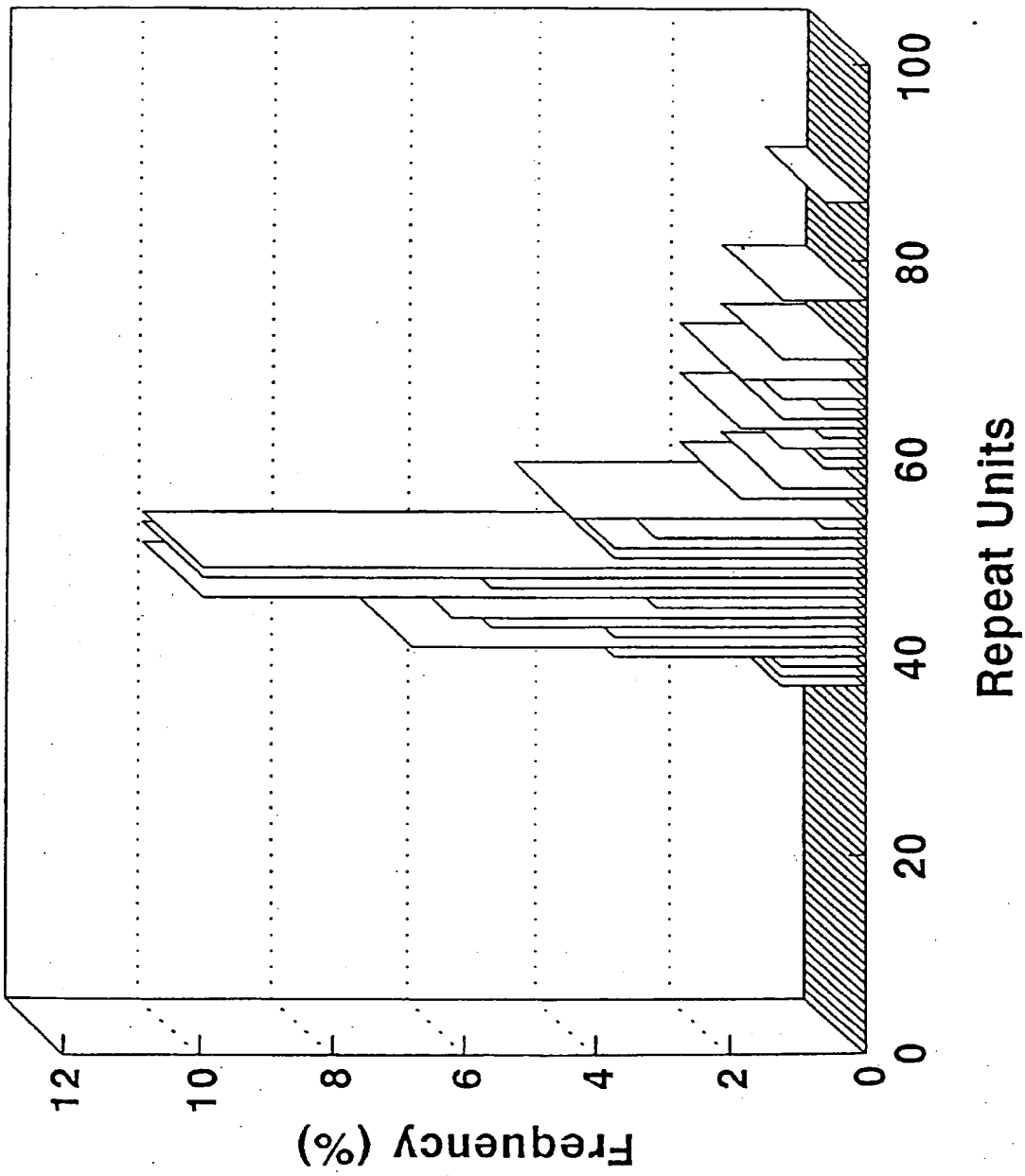


FIGURE 12 cont. (PANEL B)

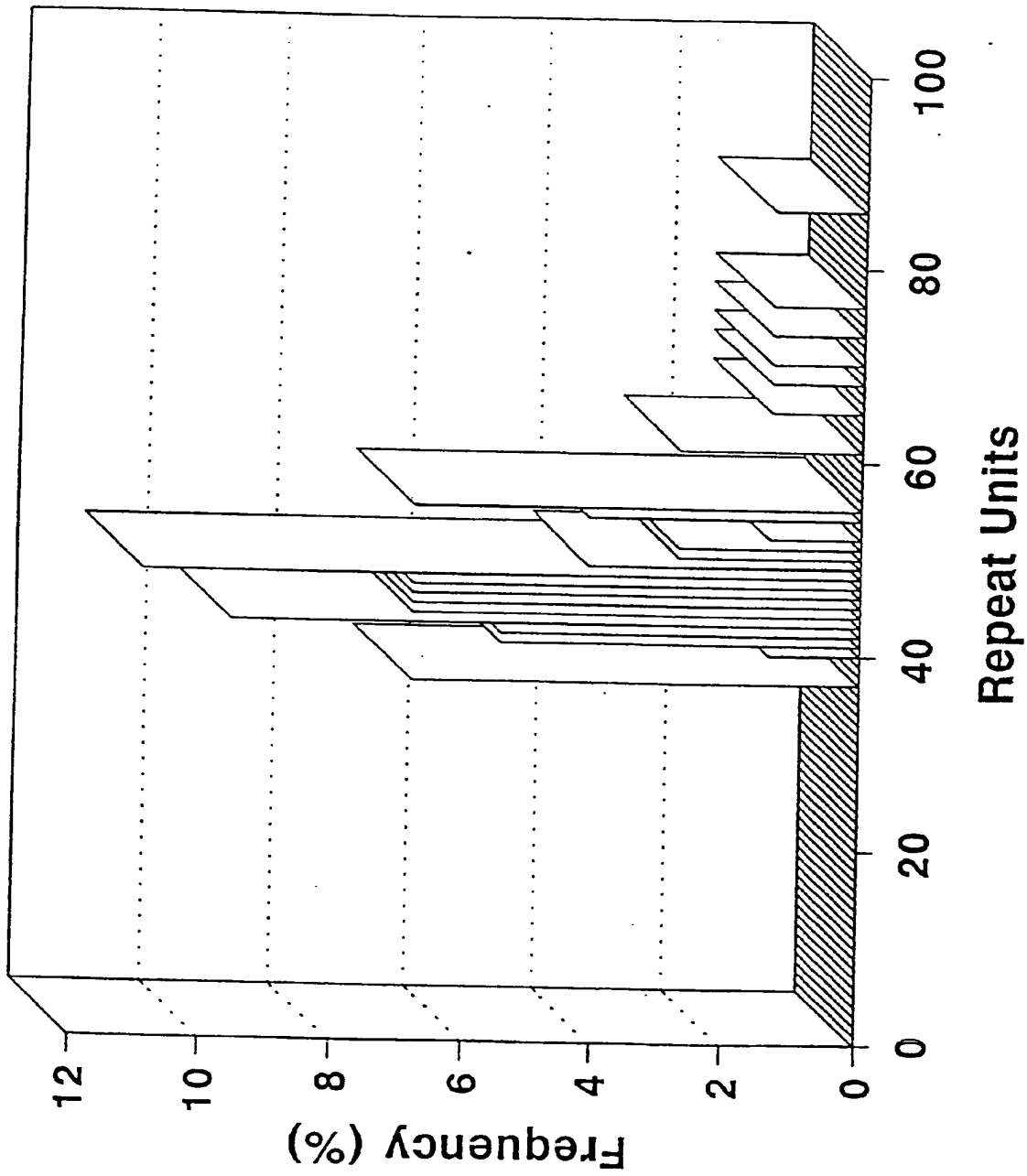
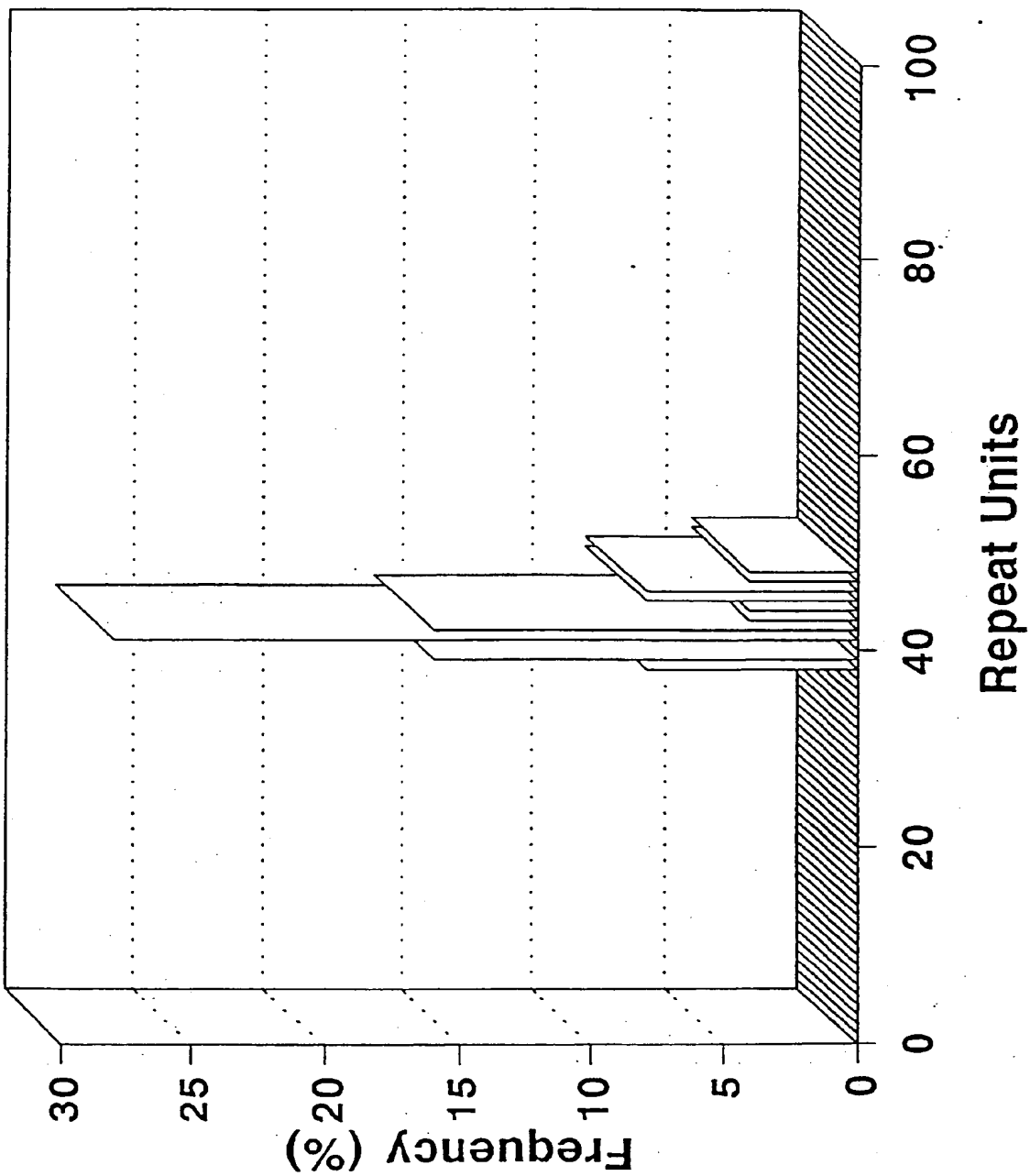


FIGURE 13 (PANEL A)



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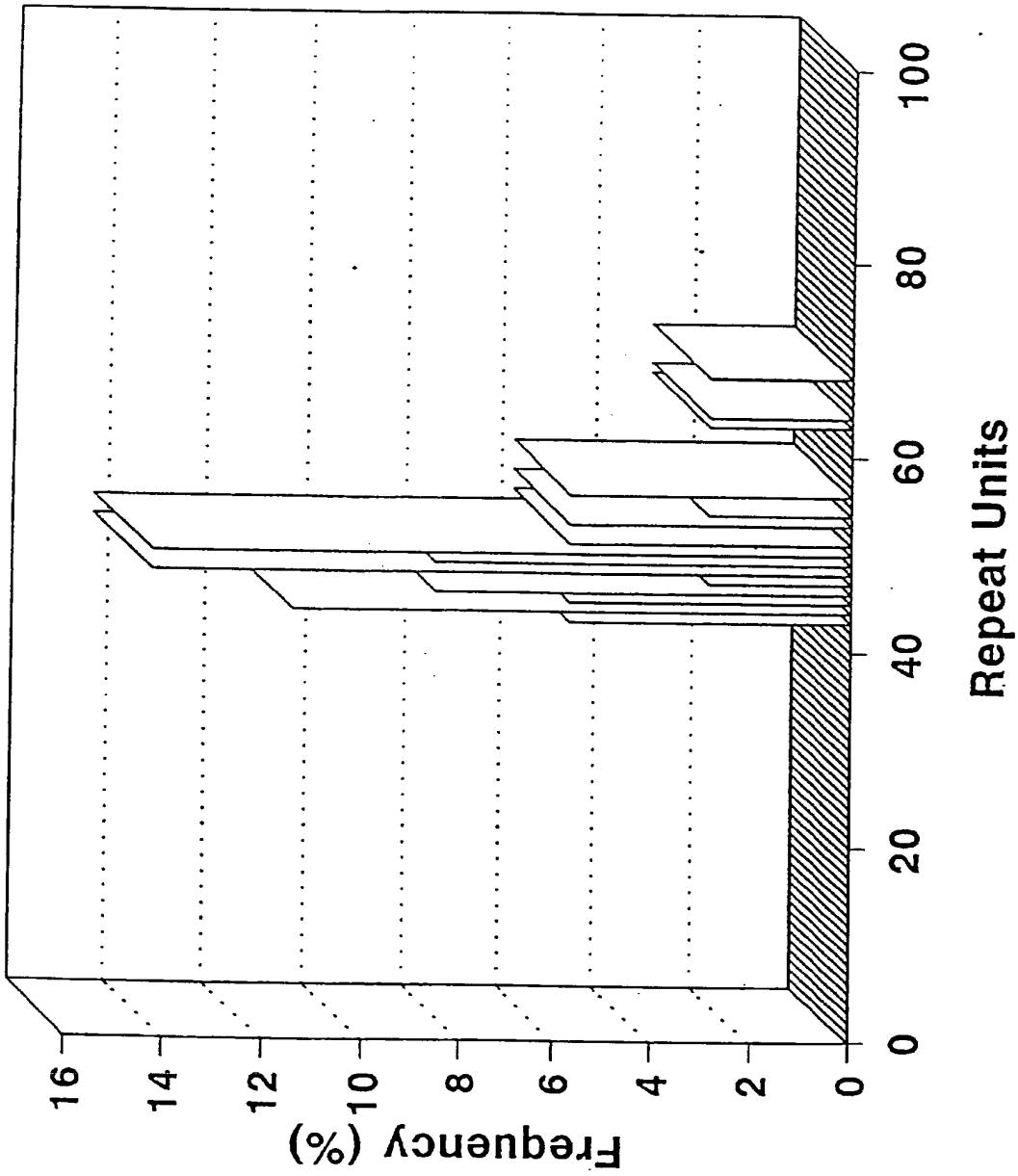


FIGURE 13 cont. (PANEL C)

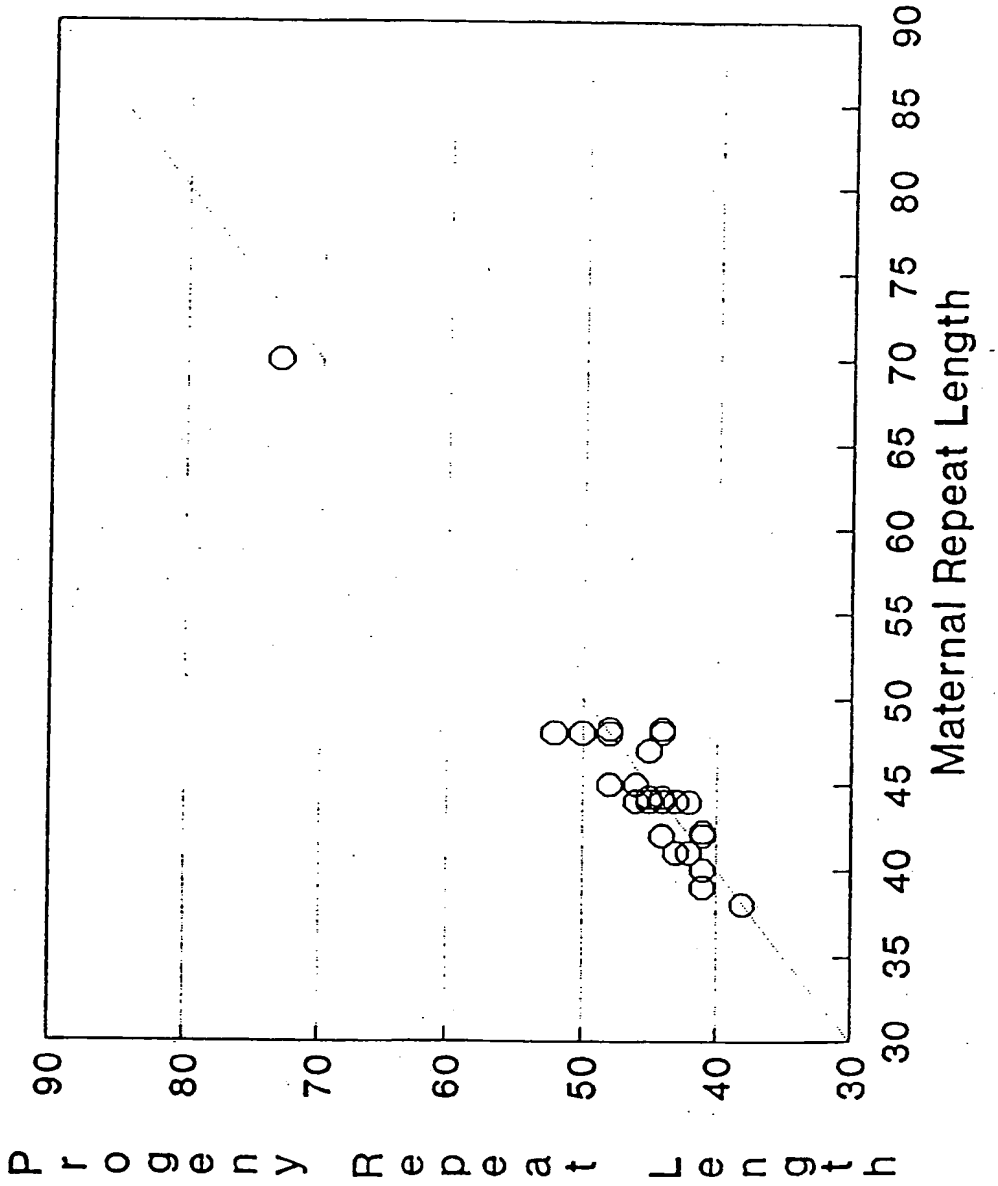


FIGURE 14 (PANEL A)

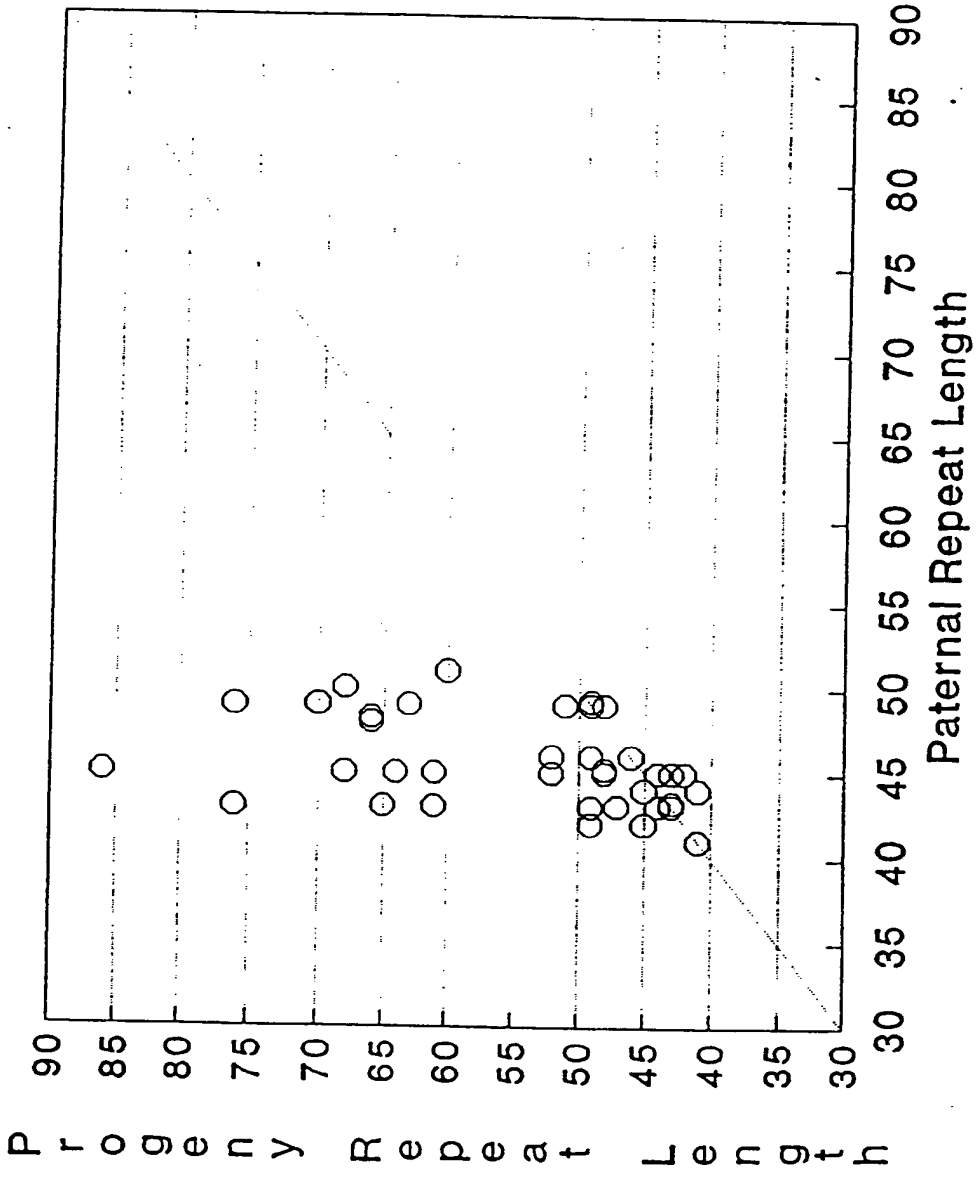
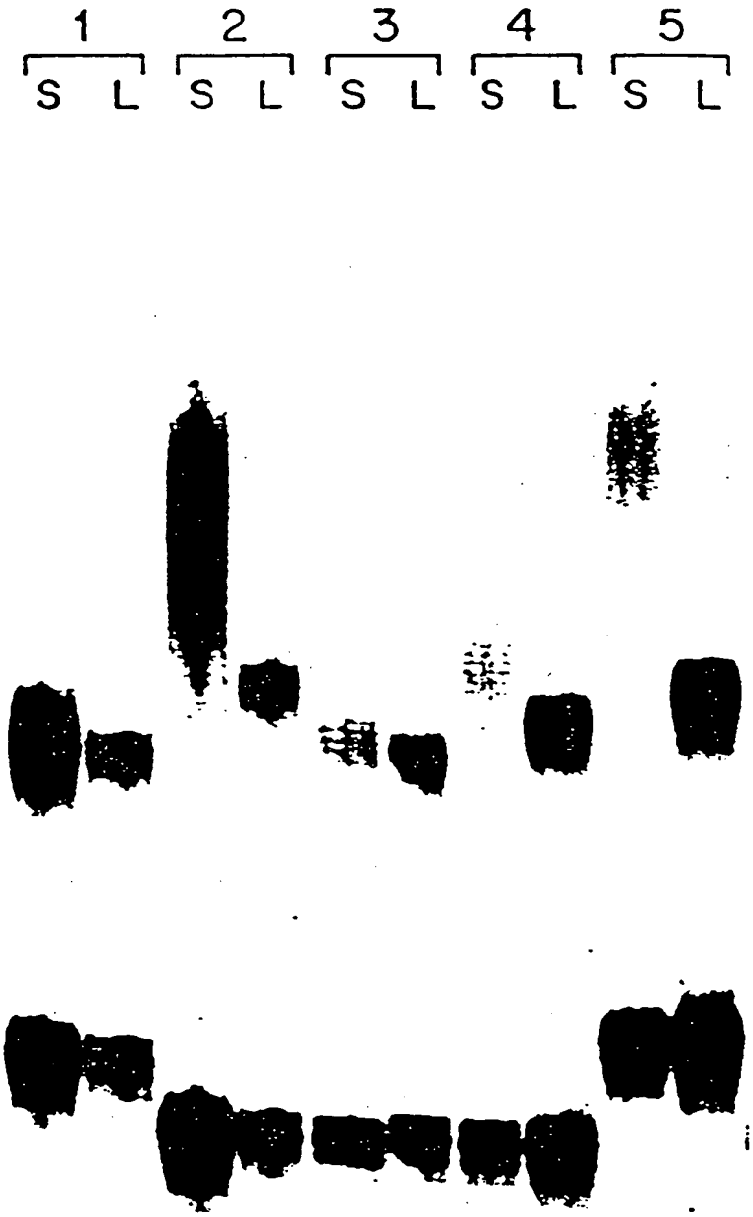


FIGURE 14 cont. (PANEL B)

FIGURE 15



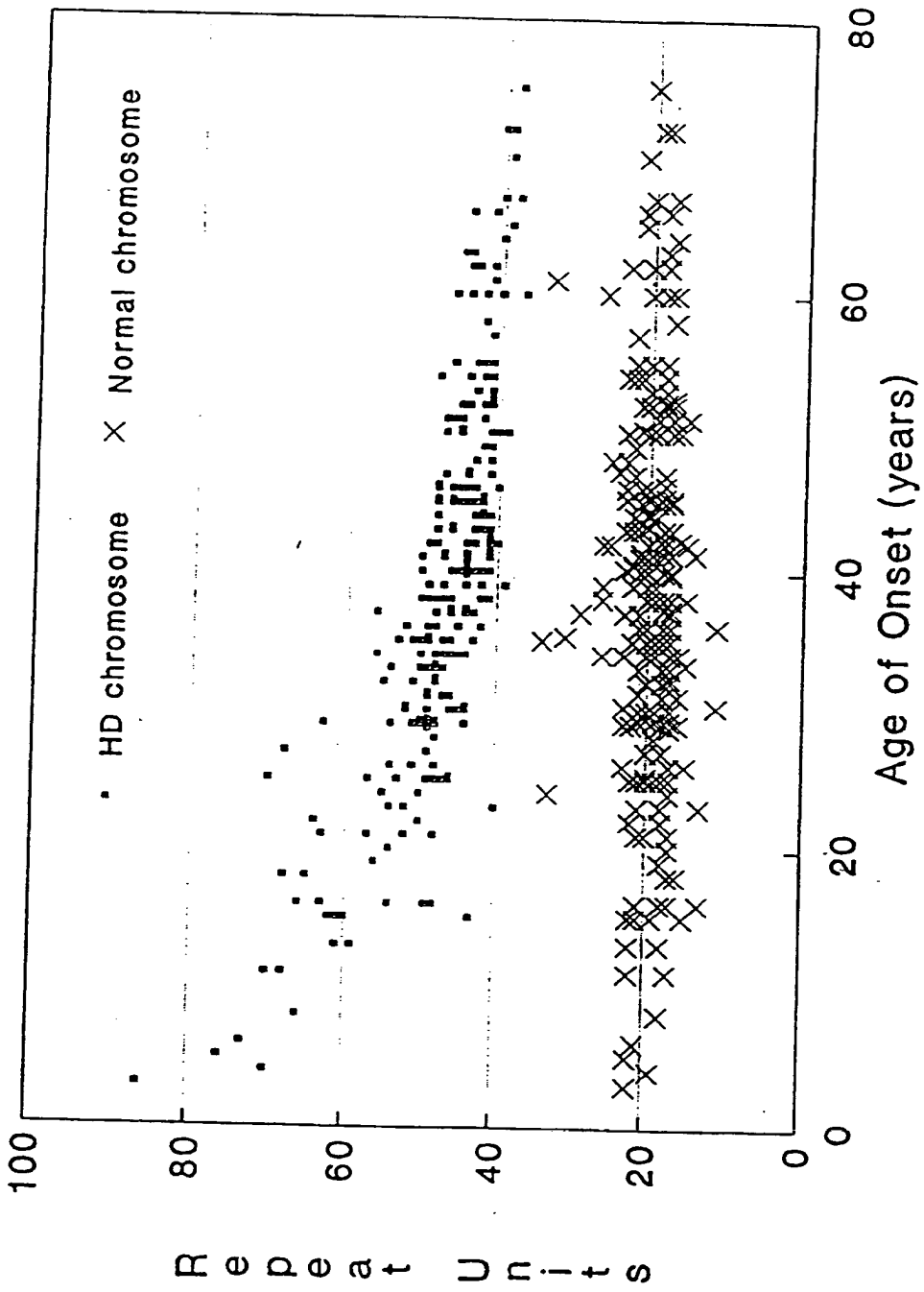


FIGURE 16

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 0 614 977 A3

(12) EUROPEAN PATENT APPLICATION

(88) Date of publication A3:
28.02.1996 Bulletin 1996/09

(43) Date of publication A2:
14.09.1994 Bulletin 1994/37

(21) Application number: **94301587.5**

(22) Date of filing: **07.03.1994**

(51) Int Cl.[6]: **C12N 15/12**, C07K 13/00, C12N 1/21, C12N 5/10, C07K 15/28, C12N 5/16, C12Q 1/68, A61K 37/02, A61K 48/00, C12P 21/08

(84) Designated Contracting States:
AT BE CH DE DK ES FR GB GR IE IT LI LU MC NL PT SE

(30) Priority: **05.03.1993 US 27498**
01.07.1993 US 85000

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London WC1N 2DD (GB)

(54) Huntingtin DNA, protein and uses thereof

(57) A novel gene, *huntingtin*, is described, encoding huntingtin protein, recombinant vectors and hosts capable of expressing huntingtin. Methods for the diagnosis and treatment of Huntington's disease are also provided.

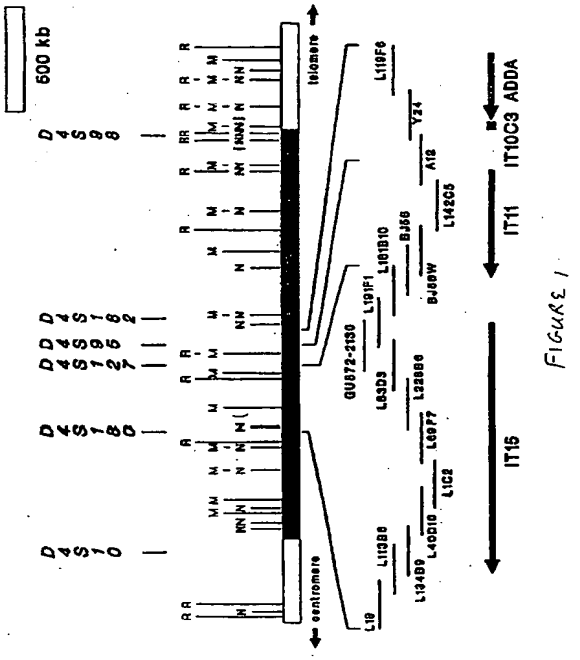

FIGURE 1

EP 0 614 977 A3

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 94 30 1587

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.5)
X,D	SOMAT. CELL MOL. GENET., vol. 17, no. 5, 1991 pages 481-488, LIN ET AL. 'New DNA markers in the Huntington's disease gene candidate region' * the whole document * ---	4,6	C12N15/12 C07K13/00 C12N1/21 C12N5/10 C07K15/28 C12N5/16 C12Q1/68 A61K37/02 A61K48/00 C12P21/08
X,D	NATURE GENET., vol. 1, May 1992 pages 99-103, MAC DONALD ET AL. 'The Huntington's disease candidate region exhibits many different haplotypes' * the whole document * ---	4,6	
P,X	CELL, vol. 72, 26 March 1993 pages 971-983, THE HUNTINGTON'S DISEASE COLLABORATIVE RESEARCH GROUP 'A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes' * the whole document * ---	1-6,8, 13-15	
P,X	CR ACAD. SCI. III, vol. 316, no. 11, November 1993 pages 1374-1380, DODÉ ET AL. 'Huntington's disease in French families : CAG repeat expansion and linkage disequilibrium analysis' * the whole document * --- -/--	4,6, 13-15	

TECHNICAL FIELDS SEARCHED (Int.Cl.5)
C07K
C12N

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	15 December 1995	Gac, G

CATEGORY OF CITED DOCUMENTS
X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
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T : theory or principle underlying the invention
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EUROPEAN SEARCH REPORT

Application Number
EP 94 30 1587

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.5)
P,X	MOL. CELL PROBES, vol. 7, no. 3, June 1993 pages 235-239, WARNER ET AL. 'A new polymerase chain reaction (PCR) assay for the trinucleotide repeat that is unstable and expanded on Huntington's disease chromosomes' * the whole document * ---	4,6, 13-15	
A	US-A-4 666 828 (GUSELLA) 19 May 1987 * the whole document * ---	1-23	
A	MOL. CELL. BIOL., vol. 10, no. 11, November 1990 pages 5616-5625, LAURENT ET AL. 'The SNF5 protein of Saccharomyces cerevisiae is a glutamine- and proline-rich transcriptional activator that affects expression of a broad spectrum of genes' * page 5618 - page 5619 * -----	19	
			TECHNICAL FIELDS SEARCHED (Int.Cl.5)

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	15 December 1995	Gac, G

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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